Università
Ca'Foscari
Venezia

Scuola Dottorale di Ateneo
Graduate School

Dottorato di ricerca
in Scienze Ambientali
Ciclo XXVII
Anno di discussione 2015

Bacterial epibiosis on the invasive seaweed *Caulerpa cylindracea* (Sonder) in the Mediterranean Sea: potential ecological implications

SETTORE SCIENTIFICO DISCIPLINARE DI AFFERENZA: BIO/07
Tesi di Dottorato di Lucia Rizzo, matricola 955955

Coordinatore del Dottorato

Prof. Gabriele Capodaglio

Tutore del Dottorando

Prof.ssa Simonetta Fraschetti

Prof.ssa Patrizia Torricelli

Co-tutore del Dottorando

Dott.ssa Loredana Stabili
Prof. Pietro Alifano
Prof. Antonio Pusceddu

TABLE OF CONTENTS

Abstract

In the Mediterranean Sea, biodiversity is strongly threatened by biological invasions. Some introduced seaweeds often have the potential to change sediment biogeochemistry leading to profound alterations into the functioning of ecosystems. *Caulerpa cylindracea* (Sonder) is a non-indigenous invasive macroalga widely distributed in all Mediterranean habitats, representing a serious threat for native assemblages. The seaweed can harbour microbial communities and, in turn, epiphytes could be a source of bioactive compounds. In the present thesis, *C. cylindracea* was collected in Torre Guaceto (Brindisi, Italy) on the rocky shallow substrates in four sampling times in order to study the associated epibacterial community. Cultured and uncultured bacteria have been compared to describe the microbial community associated to *C. cylindracea*. Gamma-Proteobacteria belonging to the genera *Shewanella* and *Vibrio* have been found by both approaches on the algal surface consistently in time, along with an unknown species belonging to the Rhodobacteraceae. Other taxa belong to *Bacillus*, *Pseudoalteromonas*, *Tropicibacter*, *Photobacterium*, *Exiguobacterium*, *Kocuria*, *Ruegeria* and *Marinobacter* genera have been discovered with the culturable approach. The isolated *Vibrio* were identified to species level. Most of the observed *C. cylindracea*-*Vibrio* associations were consistent in time, by contrast, only few seem to be sporadic. Moreover, the cultural and molecular approaches were used to analyze the microbial metabolic profiles associated to the surface of the seaweed collected in different sites across the central Mediterranean Sea. These results showed that there is a distinct group of bacteria consistently present on *C. cylindracea*, irrespective of its geographical origin. Finally, in the five sites, the microbial metabolic patterns associated to *C. cylindracea* surface, as well as to sediments uncolonized and colonized by *C. cylindracea*, were compared. Moreover, in the same sites the biochemical composition of the organic matter was analysed both in sediment invaded and not-invaded by *C. cylindracea*. Results revealed greater organic matter concentration in seaweed colonized sediments than in seaweed uncolonized ones. In conclusion, *Caulerpa cylindracea* appears to host a specialized bacterial community with

a functional diversity potentially playing a crucial role in the biogeochemistry of the sediment and in the patterns of algal invasions.

Riassunto

Nel Mar Mediterraneo la biodiversità è fortemente minacciata dalle invasioni biologiche. Spesso le alghe introdotte hanno la capacità di modificare la biogeochimica del sedimento determinando profonde alterazioni nel funzionamento degli ecosistemi. *Caulerpa cylindracea* (Sonder) è una macroalga non-indigena invasiva ampiamente distribuita in tutti gli habitat del Mediterraneo dove rappresenta una seria minaccia per le comunità locali. Le alghe possono ospitare diverse comunità microbiche che, a loro volta, possono avere un ruolo protettivo nei confronti dell'alga. In questa tesi, *C. cylindracea* è stata raccolta presso Torre Guaceto (Brindisi, Italia) nell'infralitorale roccioso in quattro date di campionamento, al fine di analizzare la comunità epibatterica associata mediante l'utilizzo di metodiche colturali e molecolari. Sulla superficie di *C. cylindracea*, in tutti i tempi analizzati, sono stati identificati con entrambi i metodi Gamma-Proteobacteria appartenenti ai generi *Shewanella* e *Vibrio*, insieme ad un taxon appartenente alle Rhodobacteraceae. Altri generi *Bacillus*, *Pseudoalteromonas*, *Tropicibacter*, *Photobacterium*, *Exiguobacterium*, *Kocuria*, *Ruegeria* e *Marinobacter* sono stati isolati utilizzando metodi colturali e i vibrioni sono stati identificati a livello di specie. Molte associazioni tra *C. cylindracea* e il genere *Vibrio* sono state osservate costantemente, mentre solo pochi vibrioni sembrano essere sporadici. L'indagine sulla comunità batterica associata alla superficie dell'alga invasiva è stata condotta anche in cinque siti del Mediterraneo centrale, mediante l'uso integrato di metodi colturali e molecolari. I dati ottenuti confermano l'esistenza di un gruppo distinto di batteri presente su *C. cylindracea*, indipendentemente dalla località campionata. Nei cinque siti sono stati comparati i *pattern* metabolici delle comunità microbiche associate alla superficie di *C. cylindracea* con i *pattern* metabolici delle comunità microbiche presenti nel sedimento proveniente da aree colonizzate e non colonizzate dall'alga invasiva. Inoltre è stata analizzata la composizione biochimica della sostanza organica nei sedimenti, rivelando una maggiore concentrazione di sostanza organica nei sedimenti colonizzati dall'alga. In conclusione, *Caulerpa cylindracea* sembra ospitare una comunità batterica specializzata, con una

diversità funzionale che potrebbe giocare un ruolo cruciale nella biogeochimica dei sedimenti e nei *pattern* di invasione algali.

1. General Introduction

1.1. Invasive species in the Mediterranean Sea

The Mediterranean Sea is considered a marine hotspot of biodiversity thanks to particular biogeochemical conditions of the basin; nevertheless it is exposed to several threats such as habitat loss and degradation, fishing impacts, pollution, climate change, eutrophication and biological alien invasion (Coll et al., 2010; Claudet and Fraschetti, 2010). It is known that the introduced seaweeds can affect habitat complexity and change sediment biogeochemistry (Lorenti et al., 2011). The introduction of potentially habitat forming species, such as non-indigenous species, may represent both direct and indirect source of variation, leading to a multiplicity of effects (Neira et al., 2006). Some studies reported that introduced species lead to biodiversity loss (Vitousek et al., 1996; Wilcover et al., 1998). Introduced species, causing native marine communities changes or negative economic impacts, are considered invasive species. A recent review about non-indigenous species has highlighted the presence of 986 marine species in the Mediterranean basin, the list can be consulted in the Marine Mediterranean Invasive Alien Species (MAMIAS) database (UNEP RAC/SPA, 2012). Since 2011, 48 new entries are reported: the rate of new introduction has been approximately estimated at one new entry every two weeks (Zenetos et al., 2012). The dominant groups among alien species are molluscs, crustaceans, polychaetes macrophyta and fish (Zenetos et al., 2010; Zenetos et al., 2011). In particular, macrophytes are approximately 30% of all non-indigenous species in the Adriatic Sea and in the Western Mediterranean Sea, representing the principal group in these areas (Zenetos et al., 2012). Among invasive seaweeds, siphonous green seaweeds are able to compete directly with native species and, once introduced, they became successful invaders (Williams et al., 2007).

The most important vectors for seaweed introductions are fouling of vessels, activity related to aquaculture and ballast water. Williams et al. (2007) have reviewed globally ecological effects of introduced seaweeds and have highlighted generally negative trends. Invasive species could weaken indigenous communities, they are able to change biotic

and abiotic conditions, moreover they can spread pathogens (Strayer et al., 2006; Ricciardi and Cohen, 2007; Thomsen et al., 2009; Stevenson et al., 2012).

The evidences suggest the imminent need to prevent seaweed introductions taking in account that they are extremely difficult and expansive to eradicate (Lodge et al., 2006; Schaffelke et al., 2006).

Several introduced macrophytes could modify the ecosystem structure and functions, acting as ecosystem engineers (Thresher, 2000, Bianchi, 2007).

Globally, a great part of the introduced green algae are siphonous, belonging to the order Bryopsidales. They are all unicellular and composed of elementary multinucleate tubes, as occurs in genus *Bryopsis*, or complex network of interconnected tubes with millions of nuclei, as occurs in genus *Caulerpa* (Williams et al., 2007). The siphonous algae have several ability: they can occupy several type of habitats, grow rapidly, spread though fragmentation and propagation (Wright and Davis, 2006) and in addition are able to recover by injures (Dreher et al., 1978). These characteristics help great success of many invasive species, mainly *Caulerpa* spp. (Smith and Walters, 1999).

1.2. Species under study: *Caulerpa cylindracea* (Sonder)

In the Mediterranean Sea, invasive species belonging to genus *Caulerpa* (Caulerpales, Chlorophyta) represent one of the main threats to natural marine environments (Meinesz et al., 2001), in particular, they are *Caulerpa taxifolia* (Vahl) C. Agardh and *Caulerpa cylindracea* (Sonder) (Belton et al., 2014), previously known as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman and Boudouresque (Verlaque et al., 2003). Algae belonging to genus *Caulerpa* are particularly worrying because they are able to spread over all substrates, competing with native species and overgrowing benthic indigenous assemblages (Ceccherelli and Piazzi, 2001; Piazzi et al., 2005a), both utilize the same strategy because they are able to lengthen rapidly during the period of maximal growth (Piazzi and Cinelli, 1999). Moreover *C. cylindracea* reproduces by propagules (Renoncourt and Meinesz, 2002) and sexually (Panayotidis and Zuljevic, 2001), achieving even higher speed than *C. taxifolia* (Piazzi et al., 2005b) which in the Mediterranean Sea does not reproduce sexually (Carvalho et al., 1998).

Caulerpa cylindracea was considered by Lipkin (1975) a Lessepsian species migrated from the Suez canal to the basin (Hiddink et al., 2012) in the Mediterranean Sea. During the 1990s, a new taxon of *Caulerpa* was observed in the seas of Libya (Nizamuddin, 1991), related to Western Australian taxon (Fama' et al., 2000) and identified as *C. cylindracea* Sonder. Morphologically, *C. cylindracea* has erect fronds with sparse vesiculate ramuli (Fig. 1.1) and the stolon is fixed to the substrate by thin short rhizoids (Verlaque et al., 2003). Some studies documented seasonal variations of stolon and erect axis length, growth rate, cover and biomass in *C. cylindracea*, with maximum biomass, frond height, stolon length in September-November (Piazzi and Cinelli, 1999; Piazzi et al., 2001). Regional variations observed depend probably on different experimental conditions and methodological approaches (Capiomont et al., 2005; Ruitton et al., 2005).

Caulerpa cylindracea produces secondary metabolites, such as caulerpenyne, in order to defend itself from other species. In addition, *C. cylindracea* possess an antioxidant system that is able to transform the oxygen radicals into water and molecular oxygen (Cavas and Yurdakoc, 2005). However, even if *C. cylindracea* possesses several defence systems (Jung et al., 2002; Dumay et al., 2002; Cavas et al., 2006), several sea urchins and fishes feed on *C. cylindracea* (Azzurro et al., 2004; Ruitton et al., 2006; Felline et al., 2012).

It has exhibited invasive peculiarity colonizing all available habitats and substrates and leading to profound alterations of indigenous assemblages (Holmer et al., 2009; Cebrian et al., 2012), probably supported by temperature increasing in the basin (Lejeusne et al., 2010).

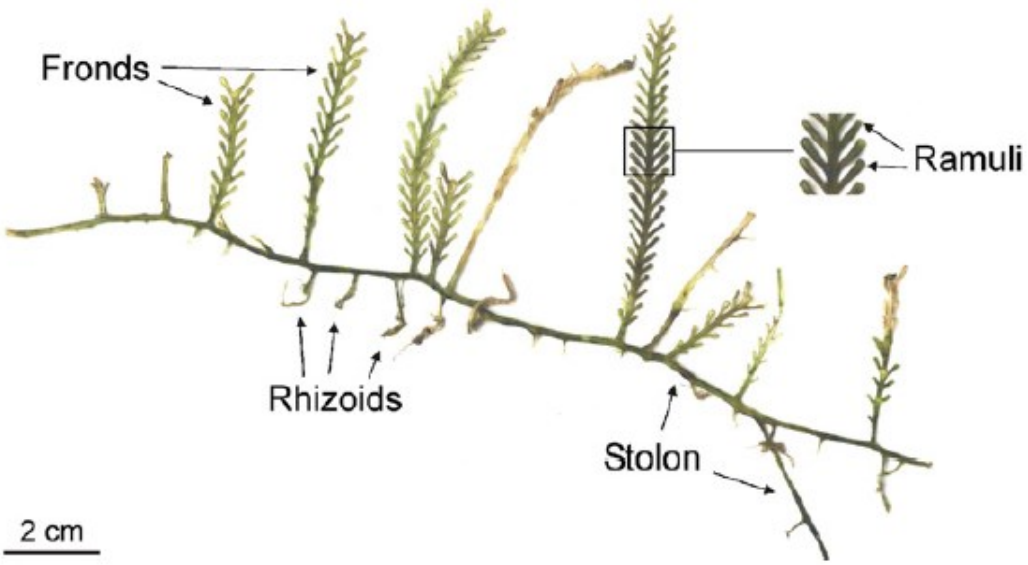


Figure 1.1. Thallus of the invasive *C. cylindracea*. Herbarium specimen, J. Klein. (Klein and Verlaque, 2008).

Monospecific meadows of *C. cylindracea* were not observed in the home range of the species corresponding to south-western Australia (Carruthers et al., 1993; Verlaque et al., 2003), contrarily, in the Mediterranean Sea, *C. cylindracea* forms continuous dense meadows in several habitats. It is able to inject in sparse *Posidonia oceanica* meadows (Panayotidis and Montesanto, 1994; Piazzi et al., 1997a,b; Serio and Pizzuto, 1998; Ceccherelli and Piazzi, 1999; Piazzi and Cinelli, 1999; Ceccherelli et al., 2000, Ceccherelli and Piazzi, 2001; Zuljevic et al., 2004; Tsirika and Haritonidis, 2005), while a good state of health meadows hinder the penetration (Ceccherelli et al., 2000). Turf and encrusting species seem to help *C. cylindracea* while erect algae prevent the spread of the invasive seaweed (Ceccherelli and Campo, 2002).

Taking in account its ecological impact and fast spread, *C. cylindracea* has been inserted in the 100 worst invasive species of the Mediterranean Sea (Streftaris and Zenetos, 2006). In addition, the loss of beta diversity and the homogenization of the Mediterranean coastal system observed in invaded site lead to hypothesize a role of ecosystem engineer (Pacciardi et al., 2011).

It is known that the introduced seaweeds can affect habitat complexity and change sediment biogeochemistry (Lorenti et al., 2011). The introduction of potentially habitat forming species, such as non-indigenous species, may represent both direct and indirect source of variation, leading to a multiplicity of effects (Neira et al., 2006); in particular

the impact on the faunal assemblages depends on several factors including the type of habitat invaded, the time passed since the introduction (Neira et al., 2005) and the sediment fraction affected by the invasion (McKinnon et al., 2009).

Investigations of the effects of *C. cylindracea* on soft bottom macrofauna have been conducted on several assemblages and habitats and have highlighted that generally, community associated to *C. cylindracea* present higher biodiversity and abundance than bare substrates (Pandolfo and Chemello, 1995; Argyrou et al., 1999; Buia et al., 2001; Lorenti et al., 2009; Vazquez-Luis et al., 2009; Box et al., 2010; Pacciardi et al., 2011). The effects of *C. cylindracea* on indigenous benthic assemblages have been also studied on rocky bottoms (Piazzi et al., 2001; Piazzi and Balata, 2007; 2009; Klein and Verlaque, 2009), underlining negative effects of *C. cylindracea* on erect macroalgae, both articulated and foliose (Piazzi et al., 2005b). In coastal environments, sedimentation is considered an important agent of change in benthic communities (Airoldi, 2003; Claudet and Fraschetti, 2010), supporting tolerant and opportunistic species (Irving and Connell, 2002a,b; Eriksson and Johansson, 2003; Balata et al., 2005). In effect, once established, *C. cylindracea* is able to enhance sediment accumulation, supporting algal turfs to the detriment of erect macroalgae and becoming, in this way, the main drivers of ecological change (Bulleri et al., 2010; Bulleri and Benedetti-Cecchi, 2008). *Caulerpa cylindracea* is tolerant to high sedimentation rates and its competitiveness could be supported by the capacity to trap sediments (Piazzi et al., 2005a) affecting habitat complexity (Piazzi et al., 2005b). This invasive seaweed is able to compact several layers of sediment up to 15 cm thick and to modify hydrodynamics near the seabed (Argyrou et al., 1999; Žuljević et al., 2003; Piazzi et al., 2007; Hendricks et al., 2010).

Some studies have analysed the variability of biogeochemical parameters in the sediments conditioned by *C. cylindracea* (Holmer et al., 2009; Matijevic et al., 2013). Higher organic carbon, total nitrogen, total phosphorus content, microbial activity and sulfide pools of the sediments have been recorded at invaded sites compared with sites where the invasive alga was absent. Furthermore in invaded sites, organic carbon/ total nitrogen ratio, organic carbon/ total phosphorus content ratio and negative redox-potential reveal that the organic matter was originated from seagrass degradation under anoxic conditions (Matijevic et al., 2013). Considering the strong changes on habitat architecture and sediments, *C. cylindracea* can be considered as a habitat modifier (Wallentinus and Nyberg, 2007).

Several hypotheses have been formulated to understand the ability of the introduced macroalga to expand and adapt to new environmental conditions (Keane and Crawley, 2002; Torchin et al., 2003; Callaway and Ridenour, 2004). Up to now, morphological and physiological traits have been studied mainly in order to assess the potential of invasion (Facon et al., 2008; Klein and Verlaque, 2008). However, also the associated microbes, transmitted vertically and co-introduced, as well as the induced sediment modification (Klein and Verlaque, 2008; Byers et al., 2010), can determine the competitive potential of invasive species, influencing the function and evolution of their host (Adams and Kloepper, 2002). Meusnier et al. (2001) have analysed bacterial assemblages associated to the alga *C. taxifolia* native populations from Northern Australia, and non-indigenous population from Mediterranean basin and have reported that the bacterial communities were similar in both areas.

Aires et al. (2013) recently have identified and compared putative bacterial epiphytic and endophytic communities in the native and introduced populations of the marine green macroalga *C. cylindracea*, through pyrosequencing, confirming the origin of invasion. In this scenario, the potential role of bacterial communities in their eukaryotic host could be crucial for the adaptation of the species. It is important to consider introduced species and their associated micro-organisms as 'meta-organism' in order to understand mechanisms underlying the great success of such non-indigenous species.

1.3. Biofilm development, composition and dynamics

In the marine ecosystem any natural and artificial surfaces are fast settled by microorganisms and macroorganisms (Wahl, 1997). In general, four sequential, overlapped or parallel phases (Cooksey and Wigglesworth-Cooksey, 1995; Maki, 2002) of marine biofouling colonisation are identified and summarized: adsorption of dissolved organic molecules, colonisation by prokaryotes, colonisation by unicellular eukaryotes and recruitment of invertebrate larvae and algal spores (Wahl, 1997; Railkin, 2004). Some studies have demonstrated as the first two phases are crucial for the larval and spore settlement (Dobretsov et al., 2006; Qian et al., 2007).

Biofilms can hinder or help microbial settlement and provide some carbon source for microorganisms growth. Microbial attachment is affected by substratum physical properties such as the surface roughness (Fletcher and Loeb, 1979; Railkin, 2004) and the

hydrodynamic regime near the surface like water turbulence (Zobell, 1943; Costerton et al., 1995; Cao and Alaerts, 1995). Furthermore, attachment of bacteria and microalgae to substrata surfaces, may be mediated by exudate of particular extracellular polymers (EPS), similar to glue, made by polysaccharides, lipopolysaccharides, proteins and nucleic acids (Flemming et al., 2001). Chemical responses are also responsible for microorganisms adhesion allowing to the attraction or repulsion to the substrata (Marshall et al., 1971, Vandevivere and Kirchman, 1993). The first microbial biofilm can inhibit or facilitate the co-attachment of other settlers until the formation of a multispecies heterogeneous biofouling (McEldowney and Fletcher, 1986).

Many studies have highlighted how several factors, in separate or combined ways, can strongly affect the architecture as well as the three-dimensional structure (O'Toole et al., 2000) (Fig. 1.2) of mature biofouling (Stoodley et al., 2002). These factors include:
- physical factors, such as flow rate, hydrodynamic forces, substrate properties, viscosity,
- chemical factors, such as nutrient availability, EPS production,
- biological factors such as intraspecies and interspecies competition and predation.

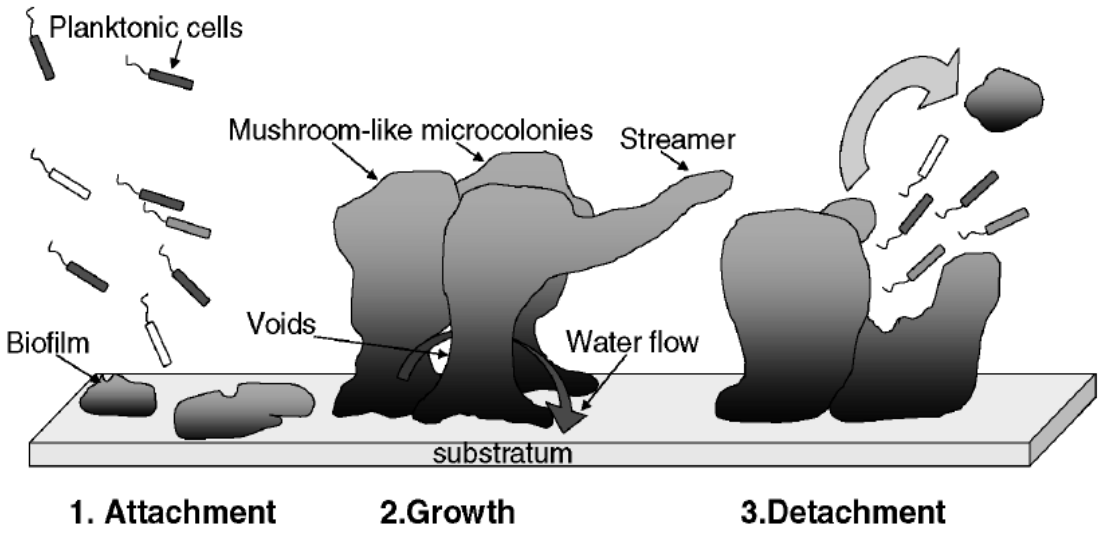


Figure 1.2. The life cycle of a biofilm: Attachment of microorganisms, formation of biofilm structure and detachment of the biofilm. (Dobretsov, 2009).

Our understanding of biofilm architecture and structure is strongly increased thanks to the development of imaging technology and scanning confocal laser microscopy (SCLM), real time image capture, atomic force microscopy (AFM), nuclear magnetic resonance imaging (NMRI) and fluorescent in situ hybridisation (FISH) methods (Dobretsov, 2009).

According to the modern model, biofilms are composed of microcolonies of bacterial cells divided by interstitial voids (Lewandowski, 2000) (Fig. 1.3). Although the shape of the microcolonies changes within a biofilm and between biofilms, the microcolonies are made up by dense layers filled with EPS and a network of interstitial voids that allow a water flow inside the biofilms (Costerton et al., 1995), conditioning their shape in relation to flow velocity (Lewandowski,2000). In general, in marine environments, microbial biofilms have very heterogenic and dynamic structures and consequently it is difficult to investigate biofouling features in a model.

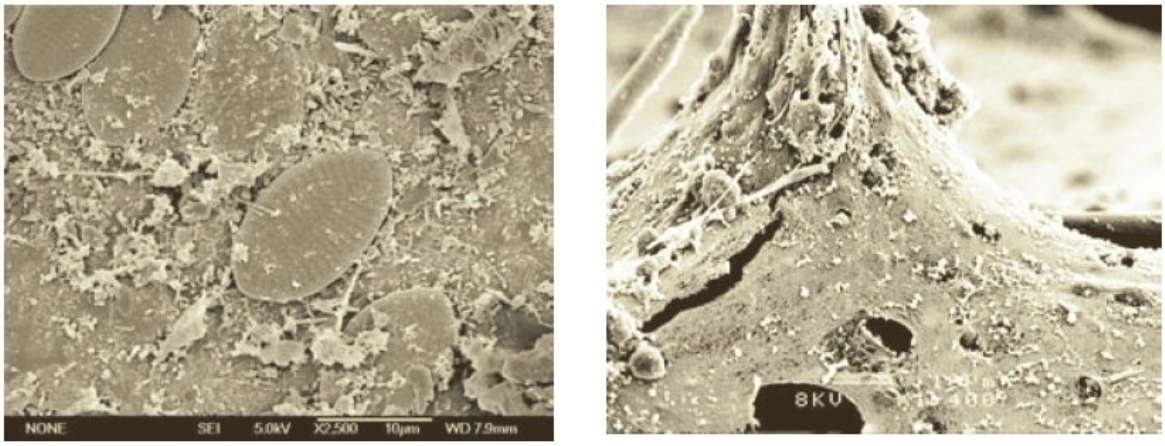


Figure 1.3. Scanning electron microphotographs of multispecies microbial biofilms developed on the surface of the alga Sargassum sp. and on the sponge Halichondria sp. (Modified from Dobretsov, 2009).

In marine biofouling the densities of other microorganisms, such as fungi, sarcodines and ciliates, are very low (Railkin, 2004) (Table 1.1), because it is mainly made up by several species of bacteria, *Archaea* and unicellular organisms.

Table 1.1 Dominant microorganisms in marine biofilms (Modified from Dobretsov, 2009).

Microorganisms	Density	Reference
Virus	10-200 x 10^7/ml	Weinbauer, 2004
Bacteria	3 x 10^6-1.3 x 10^7cell/ cm^2	Jones et al., 2007; Dovretsov and Qian, 2006; Thompson et al., 2002
Archaea	1 x 10^4 cell/ cm^2	Webster et al., 2004
Diatoms	6 x 10^3- 3 x 10^4 cell/ cm^2	Railkin, 2004; Dovretsov and Qian, 2006
Flagellates	3 x 10^3 cell/ cm^2	Railkin, 2004; Maybruck and Rogerson, 2004; Fenchel, 1986

According to Railkin (2004), the first colonisers seem to be rod-shaped bacteria, following to cocci, vibrios and spirilli-like bacteria. The last bacterial colonisers belong to the genera *Caulobacter* and *Hyphomicrobium* (Railkin, 2004). In mature marine biofilms, bacteria belonging to α- Proteobacteria, γ -Proteobacteria (41–58%; Fig. 1.4) and the *Cytophaga–Flavobacterium* group of Bacteroidetes are dominating (Webster et al., 2004; Jones et al., 2007) (Fig. 1.4). Analysis of 16S rRNA genes showed that bacterial clones from early biofilms are affiliated to the *Roseobacter* subgroup of α-proteobacteria and *Alteromonas* subgroup of γ -Proteobacteria (Dang and Lovell, 2000). Most bacterial isolates from biofilms found in Hong Kong waters belonged to the *Vibrio* and *Pseudoalteromonas* groups of γ -proteobacteria (Dobretsov and Qian, 2002; Dobretsov and Qian, 2004; Qian et al., 2007). However, using different methods, the most dominant group in marine biofilms risults α-proteobacteria (Webster et al., 2004; Dobretsov et al., 2006; Dobretsov, 2009). Finally, *Cytophaga*-like bacteria developed in biofilms from an oyster farm (Nocker et al., 2004).

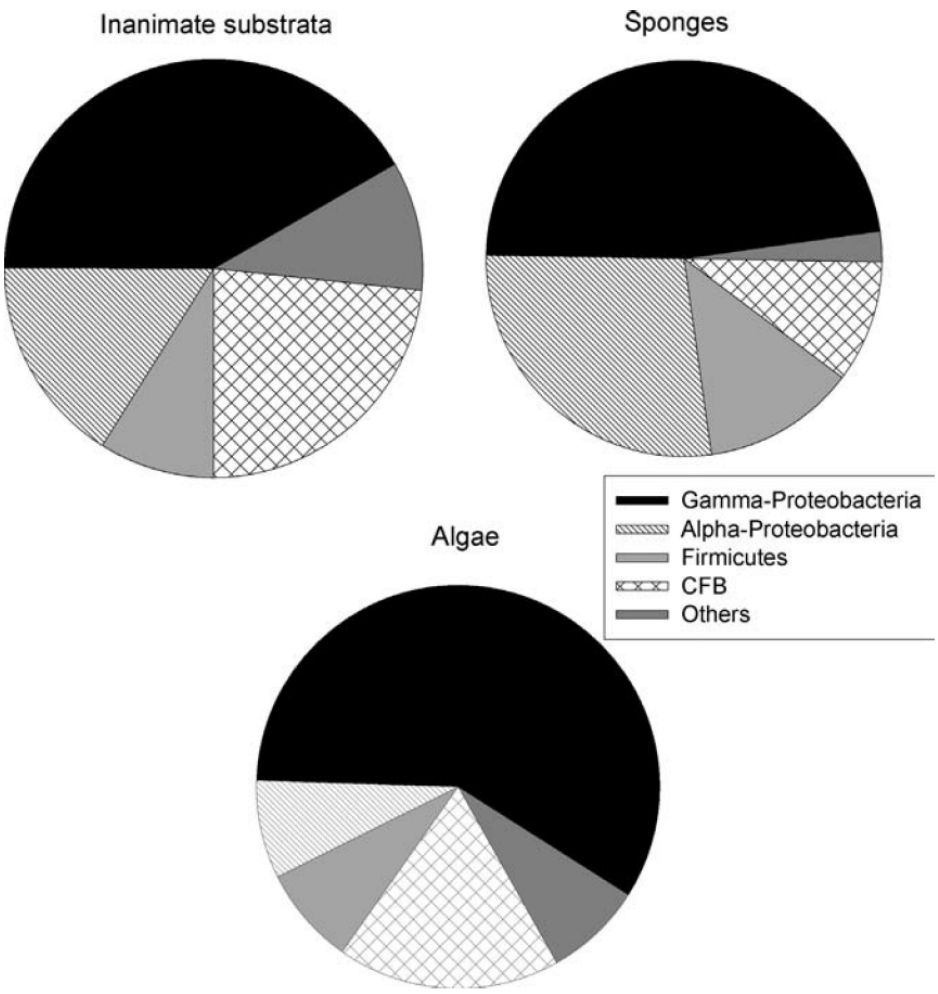


Figure 1.4. The dominant cultivated bacterial groups in biofilms from the surface of different inanimate and animate substrata. CFB, Cytophaga-Flexibacter-Bacteroides group. (Dobretsov, 2009).

Usually, in marine biofilms, species richness of marine benthic diatoms is quite high and the genera *Achnanthes*, *Amphora*, *Navicula* and *Licmophora* outnumber (Cooksey and Wigglesworth-Cooksey, 1995; Thornton et al., 2002). Fungi, mainly *Cladosporium* spp., and protists, mainly flagellates *Bodo* spp.; amoebas *Platyamoeba* spp.; infusoria *Chlamydonella* spp., can be found in low densities (Railkin, 2004; Maybruck and Rogerson, 2004; Li and Qian, 2005; Underwood, 2005) (Table 1.1). In marine biofilms, the role of fungi, protists and viruses as decomposers, grazers and parasites (Fig. 1.5) is rarely investigated. Researchers, indeed, have studied mainly monospecies microbial biofilms in static environments (Maki, 2002; Dobretsov et al., 2006), giving limited information about their role in the field and overlooking the numerous uncultivated species present in the natural biofilms (Table 1.1) (Dobretsov et al., 2006).

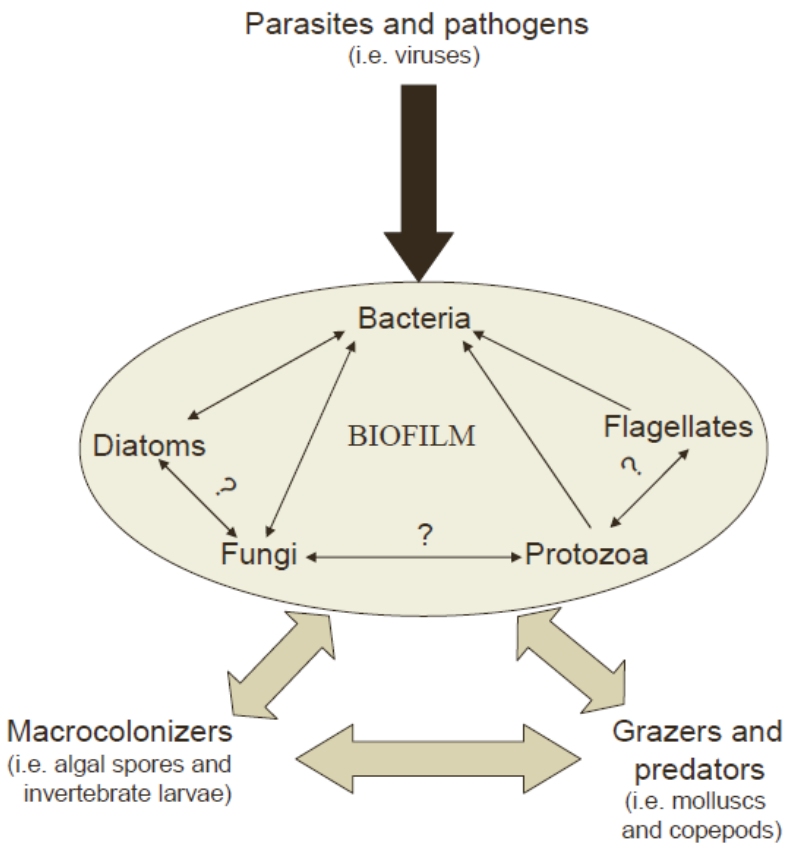


Figure 1.5. Prokaryote–eukaryote interactions in biofilms (Dovretsov, 2009).

Presence of colonisers, physical and chemical conditions of both surface and environment affect the first species that constitute earlier biofilms. Instead, mature biofilms composition, density, productivity, structure, architecture, succession rate and production of chemical compounds depend on continuous dynamic changes in abiotic and biotic components (Underwood, 2005; Qian et al., 2007). Moreover, also seasonal variations affect productivity, biomass and structure of biofilm assemblages (Forster et al., 2006; Moss et al., 2006).

Each biofilm can be considered as a "unique microhabitat", where water turbulence, temperature, amount of sediments, salinity, macroalgal canopy cover and grazer activity play an important role to determinate its properties (Lau et al., 2005; Underwood, 2005; Forster et al., 2006; Moss et al., 2006). In general, environmental conditions and intraspecific and interspecific relation can have a relevant effect on the structure, composition, physiology and function of marine biofilms.

Signalling in biofilms

Quorum sensing (QS) is a regulatory mechanism able to control biofilm growth and densities, adhesion and production. Each bacterial species uses a unique QS signal, while some species are noted to use multiple signals, released into the environment (Zhang and Dong, 2004). When bacterial density in biofilms reach a given threshold, chemical signals will bind to a receptor protein in order to transcript target genes (Dobretsov, 2009). In marine environment bacteria can produce several QS signals, however there are few knowledge about the production of QS by marine biofilms in the natural environment (Dobretsov et al., 2007). In order to properly adapt themselves to environmental condition, some microorganisms use QS signals to regulate basic cellular functions and biosynthesis of compounds, while others hinder QS signals of their competitors (Zhang and Dong, 2004). Moreover, QS signals can be also an important factors in interactions between bacteria and eukaryotes (Tait et al., 2005).

Biofilms can improve or obstruct settlement of invertebrate larvae and algal spores (Wieczorek and Todd, 1998; Maki, 2002; Qian et al., 2007) spreading chemical compound on the biofilm or in the seawater, include polysaccharides, lipids, oligopeptides, glycoconjugates, amino acids and quinines (Wieczorek and Todd, 1998; Dobretsov et al., 2006). Then, the inhibitive and inductive bacterial capacities depend on their densities and interspecific interactions with other organisms that they are establish (Dobretsov et al., 2006). The processes activing the production of these compounds are largely unknown so further investigations are need.

1.4. Prokaryote–eukaryote interactions in biofilms

Microbial biofilms are complex microhabitats where several species continuously and mutually interact (Fig. 1.5) and compete for resources and space. Interactions can be intraspecific and interspecific and they can include several types of relationships: mutualism, when organisms take benefit from the interaction; commensalism, in which only one specie takes advantages; parasitism, wherein interaction is positive for one parasite and dangerous for another host because subjected to predation, grazing, disease.

At microbiological level, some studies report positive interactions between the diatom *Navicula* sp. and the bacterium *Pseudoalteromonas* sp. (Wigglesworth-Cooksey and Cooksey, 2005), while others underline inhibitive effects of strains belonging to this

genus on larval settlement (Holmstrom, and Kjelleberg, 1999). Bacterial strains of the *Streptomyces*, *Alteromonas*, *Pseudoalteromonas* and *Roseobacter* genera are able to produce antibiotics (Dobretsov et al., 2006) in order to affect the grown of other organisms. *Pseudoalteromonas tunicata* produces antibacterial proteins to remove almost competing bacteria (Rao et al., 2005). Competitive interactions are described between bacteria and fungi in marine biofilms (Li and Qian, 2005). In general, associations in marine biofilms are very complex and it is impossible to summarise them as completely negative or positive.

Heterotrophic flagellates, together with other micrograzers, amoebas and ciliates, control the biomass, decrease the microbial diversity (Fig. 1.5) (Kiorboe et al., 2003; Maybruck and Rogerson, 2004) and increase the bacterial EPS production (Joubert and Wolfaardt, 2006). It is known that larval settlement of the polychaete *Hydroides elegans* is reduced as well as bacterial composition and density is decreased when abundance of copepods increases in biofilms (Dahms and Qian, 2005), thus suggesting grazers and predators might act indirectly on the dynamic system of biofilm assemblages.

1.5. Epibiosis

In the marine environment only certain biological surfaces resist bacterial colonization for more or less extended periods, whereas, as a rule, bacteria rapidly colonise both living and non living submerged surfaces. (Wahl, 2009).

In aquatic ecosystem, and especially in marine environment, submerged living surfaces, represented by sessile organisms, are abundant since sessile lifestyle is largely represented by all sponges, most cnidarians, many mollusca, some rotifers, most tentaculata, the tube-building polychaetes, some echinoderms and most tunicates. Sessile organisms and plants, which compete for settlement space, have developed another life strategy called epibiosis.

Epibiosis is the interaction between an organism, called basibiont that function as substratum and a sessile organism, called epibiont, attached on the basibiont surface. Epizoans and epiphytes are animal and alga epibionts, respectively (Araujo et al., 1992). Epibiotic communities on living substrata and biofouling communities on non-living hard substrata are very similar (Wahl, 2009). Epibiosis has a capacity to modify the basibiont's

characteristics with beneficial, neutral or detrimental context specific effects (Costerton et al., 1987; Thevanathan et al., 2000).

Epibacterial biofilms affect the flux of compounds for basibiont interacting on metabolically processes (Turnes et al., 1992). Bacterial biofilms, diatoms, fungi and protozoans change living interface at chemical level: for instance some microorganisms should modify concentrations of important element, such as O_2, CO_2, H^+ and nutrients (Araujo et al., 1992; Thevanathan et al., 2000). Furthermore, thickness or strong pigmentation of biofilm can obstruct light passage on basibiotic alga's surface (Costerton et al., 1987), however the basibiont can takes advantage if the new aspects discourage its consumers, other detrimental epibionts or pathogens (Turnes et al., 1992).

Epibionts like some bacteria, hydrozoans, actinians and ascidians can protect the basibiont from gastropods (Cerrano et al., 2001;Marin and Belluga, 2005), urchins (Wahl and Hay, 1995), starfish (Laudien and Wahl, 1999; Laudien and Wahl, 2004; Marin and Belluga, 2005), crabs (Wahl and Hay, 1995; Wahl et al., 1997), or fishes (Manning and Lindquist, 2003). Epibiont life is strictly dependent by basibiont.

The interaction epibiont-basibiont is very complex, it may be detrimental, tolerable or favourable and the consequences of epibionts on its host depend on nature of epibiont, on environmental conditions and the other species involved (Wahl, 1997). If epibiont is more tempting than the basibiont it'll became more vulnerable and vice versa (Wahl and Hay, 1995).

The best condition of some bacteria and diatoms is found on solid/liquid interface where nutrients are abundant. The basibiont could create particular conditions at the living surface, so epibiosis represent a facultative way to profit to exchanges linked to the basibiont's primary metabolism or of the production and excretion of secondary defence metabolites.

The effects of epibiosis are numerous, complex:variable ecological and seasonal context, the life history of a species will determine the fate of the associations.

The occurrence of epibionts varies among body parts of a basibiont, between habitats, and in time.Big patches of biofouling on body surface which exchange of nutrients, gases or light are not tolerated by basibiont, they can lose their functionalities.

A successful epibiont aim to reach reproductive maturity, then it should be able to cooperate with the basibiont's life style.

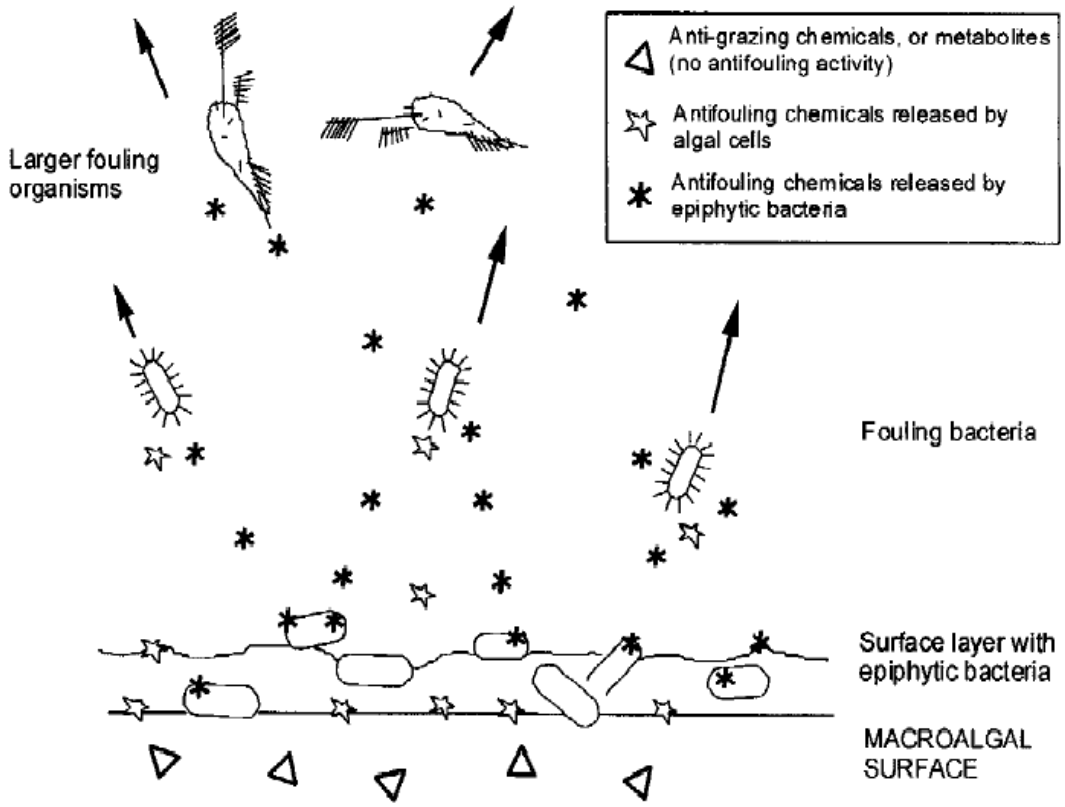


Figure 1.6. Symbiotic relationship between macroalgae and epibacteria: they can control biofilm formation (Armstrong et al., 2001).

Despite their potential important role of interactions between epibiotic bacteria and organisms in marine ecology, studies are often limited and circumstantial. A wide range of epibacterial densities, from 0 up to 10^8 cells per cm^2 is reported from the surface seaweed (Johnson et al., 1991; Rosowski, 1992), Porifera (Santavy and Colwell, 1990), Cnidaria (Stabili et al., 2006), Bryozoa (Walls et al., 1993). Bacteria, adopting adhesive strategies, live longer than the free-living forms in seawater, because they aren't exposed to the dramatic fluctuations of the environment, thanks to the microhabitat created by the hosts (Roszak and Colwell, 1987; Carman and Dobbs, 1997). These epibionts, in turn, release protective compounds into the surrounding seawater (Fig. 1.6) preventing extensive formation of biofilm on the host surface (Armstrong et al., 2001). A high level of specificity in these relationships is observed, often metabolically linked; sometimes

specific bacteria are vertically transferred from the parental host organism to its offspring though the gametes by inclusion in the oocytes or larvae (Enticknap et al., 2006). A large number of microorganisms are associated with corals, in particular mutualistic nitrogen-fixers support the symbiotic relationship between the corals and the dinoflagellate *Symbiodinium* in the oligotrophic conditions of tropical reefs (Lema et al., 2012) and protect their host by exudation of antibacterial and antiviral compounds (Vignesh et al., 2011). The production of bioactive compounds is also very important for filter feeders since they are exposed to high concentrations of potential pathogens (Olafsen, 2001). Moreover, the production of bioactive compounds by associated bacteria was evidenced in the embryos of the lobster *Homarus americanus*: their surfaces are covered almost entirely by a unique Gram-negative bacterium, producing a highly effective antifungal against a known pathogen of crustaceans (Penesyan et al., 2010). Several bacteria find a trophic source in their host. This is the case of vibrios: the settlement of chitinous structures by *Vibrio* species is a source of food for them, since they are able to produce an extracellular chitinase (Baumann et al., 1980). Some *Vibrio* species can colonise the integument of copepods especially in fecal-polluted coastal zones (Maugeri et al., 2004). Luminous bacteria, the simplest light-emitting organisms, can survive both free-living in the water column (Nealson et al., 1984) and in association with other organisms (Baumann and Baumann, 1984). Among luminous bacteria, the species mainly studied are *Photobacterium phosphoreum*, *Vibrio fischeri*, and *Vibrio harveyi*. Pujalte et al. (1999) suggested that the association with hosts might help the survival of luminous bacteria in the marine environment.

In addition, the great metabolic diversity of microbes is useful to their survival as well as their hosts, because they can create the suitable conditions to adapt also to the extreme environments in the oceans. The symbiosis should have a deep impact on the physiology, ecology, and evolution of both species (Nishiguchi et al., 1998). Extremely inhospitable habitat, such as hydrothermal vents, are inhabited by microorganisms, where they use high concentrations of hydrogen sulphide as chemical energy to synthesize organic compounds (Childress and Fisher, 1992). The flux of energy through the vent food web depends on chemiosyntethic microorganisms, as in the case of endosymbiotic bacteria that live within the suspension feeder *Riftia pachyptila*, whose digestive tract is absent and so its nutrition is dependent on the organic matter supplied by symbionts (Stewart and Cavanaugh, 2006). The evolutionary patterns of strictly related hosts and symbionts

are often congruent even if these mechanisms of the host–symbiont specificity are poorly investigated because the most of organism–bacterial relationships are experimentally difficult to set up (Stabili et al., 2008).

Seasonal or year variabilities of epibiotic assemblages are registered (Chiavelli et al., 1993; Davis and White, 1994; Fernandez et al., 1998; Dougherty and Russell, 2005). Furthermore, recent methods to identify non-cultivable strains have allowed to report host-specific assemblages of epibacteria (Harder et al., 2003; Dovretsov and Qian, 2006). In the last years, many new bacterial species, genera and orders associated to algae have been described, suggesting that macroalgae represent a source of new bacterial taxa (Goecke et al., 2010).

1.6. Bacterial Communities on Macroalgae

Being primary producers macroalgae are essential in the marine ecosystem, able to influence sediment composition and water flow. Seaweeds represent an important habitat (Weinberger et al., 1997; Bouarab et al., 2001), in which several microorganisms and macroorganisms live in strict association with them supplying directly or indirectly on macroalgae (Thomas, 2002).

Macroalgae are particularly inclined to epibiosis phenomena (Hellio et al., 2001; Lam et al., 2008) Since their living surfaces offer great quantities of organic carbon and nutrients (Kong and Chan, 1979, Armstrong et al., 2001; Lane and Kubanek, 2008). The surfaces of macroalgae, indeed, are consistently colonized by microbial communities (Bolinches et al., 1988; Jensen et al., 1996). The type of interactions with their epiphytic can be advantageous (Wahl, 2008; Goecke et al., 2010) or lead to disease and decomposition (Largo et al., 1995; Largo et al., 1997; Largo et al., 1999; Vairappan et al., 2001; Wang et al., 2008). Algae-associated bacteria can encourage algal development (Keshtacherliebson et al., 1995; Croft et al., 2005; Croft et al., 2006; Goecke et al., 2010), affect algal morphology (Provasoli and Pintner, 1980; Nakanishi et al., 1996; Matsuo et al., 2003; Marshall et al., 2006) and settlement of algal spores (Joint et al., 2002; Joint et al., 2007; Weinberger et al., 2007), and invertebrate larvae (Dobretsov and Qian, 2002; Steinberg and de Nys, 2002; Patel et al., 2003; Huggett et al., 2008). Toxins, signalling compounds, and secondary metabolites produced by algal-associated bacteria represent a source of bioactive compounds (Egan et al., 2008).

In the last decades research interest about diversity of marine microorganisms is increased (Rappe and Giovannoni, 2003; Pernthaler and Amann, 2005). Nevertheless only few studies have investigated microorganisms such as bacteria, protists (Raghukumar et al., 1992; Correa, 1997; Armstrong et al., 2000) and Archaea (Olson and Kellogg, 2010) associated with macroalgae (Egan et al., 2008; Olson and Kellogg, 2010).

The available literature on microbial communities associated with macroalgae is based on culturable-based researches (Goecke et al., 2010; Olson and Kellogg, 2010). In fact, only some studies have investigated bacterial communities using molecular biology approaches by sequence information, identification based on phylogenetic and functional traits.

Cultivation of microorganisms may not mirror abundances of microbial populations in the natural ecosystems, because only a small part of microorganisms grows on standard media approximately 1% (Amann et al., 1995; Fuhrman and Campbell, 1998; Eilers et al., 2000). Isolates reported on algal surfaces belong to the genera *Flavobacterium*, *Bacillus*, *Vibrio*, *Pseudomonas*, and *Moraxella* (Chan and Mcmanus, 1969; Lewis et al., 1985; Bolinches et al., 1988; Egan et al., 2008).

Cultivation independent approaches are recently utilized to surpass cultivation based approaches problems (Liesack et al., 1997) sequencing predominantly 16S rRNA (Pace et al., 1986). Other useful techniques can be utilized such as fluorescent *in situ* hybridization, FISH (Amann et al., 1995), or several PCR-based fingerprinting methods (Muyzer et al., 1993; Liu et al., 1997). However, some studies using sequencing approaches have faced under-sampling bias having few sequences or no replicate samples (Hughes et al., 2001; Green and Bohannan, 2006). The other hand, fingerprinting approaches mirror better microbial but have few phylogenetic resolution (Marsh, 2005).

There are many discordances between cultivation based approaches and molecular cultivation independent analysis (Bengtsson et al., 2010; Tujula et al., 2010). For instance, genus *Pseudoalteromonas* have been frequently reported from surface of Ulvaceae algae (Egan et al., 2000; Egan et al., 2001; Dobretsov and Qian, 2002; Patel et al., 2003), but were not found by Catalytically Amplified Reporter Deposition Fluorescence In Situ Hybridization CARD-FISH (Tujula et al., 2010). In some studies, great efforts were been done to investigate the microbial diversity: up to 60,000 sequences are analysed for

individual and 22 several phyla are been found, from surfaces of four algae in coral reef ecosystem, using pyrosequencing (Barott et al., 2011).

In all the studies on macroalgae α-Proteobacteria represent the most numerous phyla, followed by Bacteroidetes, Planctomycetes, Verrucomicrobia, Cyanobacteria, and d-Proteobacteria (Friedrich, 2012). Planctomycetes sequences were identified on a certain number of algae, nevertheless they might be underrepresented (Bengtsson and Ovreas, 2010; Friedrich, 2012). The surfaces of red, brown and green algae are frequently settled by Rhodobacterales, Rhizobiales such as *Mesorhizobium* spp.), and *Cyanobacterium* spp. (Meusnier et al., 2001; Tujula et al., 2010; Barott et al., 2011; Burke et al., 2011a; Lachnit et al., 2011).

Some microbial assemblages appear host-specific and are not found in the surrounding environment (Staufenberger et al., 2008; Bengtsson et al., 2010; Burke et al., 2011a; Lachnit et al., 2011; Liu et al., 2011). This different composition suggests that macroalgae is a specific habitat (Barott et al., 2011; Lachnit et al., 2011) despite conditions of seawater. A study on the kelp species *Laminaria hyperborea* reports the presence Planctomycetes bacteria in 78% of all samples in different seasons (Bengtsson et al., 2010). Instead in the Baltic Sea, only a low percentage of 7 algal host-specific bacterial populations was observed across the investigated seasons (Lachnit et al., 2011). Furthermore, the detection of specific bacterial community is difficult because it can depend on part of the investigated algal tissues. Moreover the algal age can have a role of surface settlement, nevertheless the algal species can make the difference (Staufenberger et al., 2008; Bengtsson et al., 2010). On the other hand, several studies conclude that microbial assemblages can change significantly in the same algal species and therefore, these interactions might not be host-specific (Burke et al., 2011b). The bacterial community on *Ulva australis* shows a great variability at several spatial scales (Tujula et al., 2010; Burke et al., 2011a); as well as for the brown alga *Laminaria hyperborea* (Bengtsson et al., 2010). Considerable variability of microbial assemblages was observed over seasons on macroalgae surfaces of *Fucus vesiculosus*, *Gracilaria vermiculophylla*, and *Ulva intestinalis* (Lachnit et al., 2011).

The high variability can be explained with a great functional redundancy of microbial assemblages present on macroalgae surfaces (Burke et al., 2011b). Burke et al. (2011b) have supposed that the algal surface offers a unique habitat for microorganisms equipped

with specific functional composition of genes, not related to a particular taxonomic group, suggesting that different taxa are responsible for the core functions of the microbial community on *Ulva australis* (Burke et al., 2011a).

According to the competitive lottery model (Sale, 1979) for coral reef fish, in an ecosystem, different species with the same functions can live in the same niche driven by the stochastic chances (Sale, 1979). Burke et al. (2011a,b) have translated this model, where microbial community associated to macroalgae might be determined by stochastics mechanisms, where functional genes are more important than microbial taxonomic units (Rossello-Mora and Amann, 2001).

The model of algal colonization proposed by (Burke et al., 2011a, b) results very interesting, however it should be tested in further studies directed to identify basic functional traits in order to reveal the potential role of microbial assemblages on macroalgal surfaces.

1.7. References

Adams PD, Kloepper JW (2002) Effect of host genotype on indigenous bacterial endophytes of cotton (*Gossypium hirsutum* L.). Plant Soil 240:181–189. doi:10.1023/A:1015840224564.

Aires T, Serrão EA, Kendrick G, Duarte CM, Arnaud-Haond S (2013) Invasion Is a Community Affair: Clandestine Followers in the Bacterial Community Associated to Green Algae, *Caulerpa racemosa*, Track the Invasion Source PLoS ONE 8(7): e68429. doi:10.1371/journal.pone.0068429.

Airoldi L. (2003) The effects of sedimentation on rocky coast assemblages. Oceanogr Mar Biol An Annu Rev 41:161–236.

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169.

Araujo Jorge TC, Coutinho CMLM, Aguiar LEV (1992) Sulphate-reducing bacteria associated with biocorrosion – a review. Memorias do Instituto Oswaldo Cruz 87(3):329–337.

Argyrou M, Demetropoulos A, Hadjichristophorou M. (1999) Expansion of the macroalga *Caulerpa racemosa* and changes in softbottom macrofaunal assemblages in Moni Bay, Cyprus. Oceanol Acta 22:517–528.

Armstrong E, Rogerson A, Leftley JW (2000) The abundance of heterotrophic protists associated with intertidal seaweeds. Estuar Coast Shelf Sci 50:415–424.

Armstrong E, Yan L, Boyd KG, Wright PC, Burgess JG (2001) The symbiotic role of marine microbes on living surfaces. Hydrobiologia 461:37-40.

Azzurro E, Fanelli E, Andaloro F. (2004) Preliminary data on feeding habits of dusky spinefoot *Siganus luridus* in the Sicily Channel (Central Mediterranean). Biol Mar Mediterr 11(3):145.

Balata D, Piazzi L, Cecchi E, Cinelli. F (2005) Variability of Mediterranean coralligenous assemblages subject to local variation in sediment deposits. Mar Env Res 60:403-421.

Barott KL, Rodriguez-Brito B, Janouskovec J, Marhaver KL, Smith JE, Keeling P, Rohwer FL (2011) Microbial diversity associated with four functional groups of benthic reef algae and the reef-building coral Montastraea annularis. Environ Microbiol 13:1192–1204.

Baumann P, Baumann L, Bang SS, Woolkalis MJ (1980) Revaluation of the taxonomy of Vibrio, Beneckea, and Photobacterium abolition of the genus Beneckea. Curr Microbiol 4:127–133.

Baumann P, Baumann L (1984) Genus II. Photobacterium Beijerinck 1889. In: Kreig NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore.

Belton GS, Prud'homme van Reine, WF, Huisman JM, Draisma SGA, Gurgel CFD (2014) Resolving phenotypic plasticity and species designation in the morphology challenging *Caulerpa racemosa-peltata* complex (Caulerpaceae, Chlorophyta). J Phycol 50(1):32-54.

Bengtsson MM, Ovreas L (2010) Planctomycetes dominate biofilms on surfaces of the kelp *Laminaria hyperborea*. BMC Microbiol 10:261.

Bengtsson MM, Sjotun K, Ovreas L (2010) Seasonal dynamics of bacterial biofilms on the kelp *Laminaria hyperborea*. Aquat Microb Ecol 60:71–83.

Bianchi CN (2007) Biodiversity issues for the forthcoming tropical Mediterranean Sea. Hydrobiologia 580:7-21.

Bolinches J, Lemos ML, Barja JL (1988) Population dynamics of heterotrophic bacterial vommunities associated with *Fucus vesiculosus* and *Ulva rigida* in an estuary. Microb Ecol 15:345–357.

Bouarab K, Potin P, Weinberger F, Correa J, Kloareg B (2001) The *Chondrus crispus-Acrochaete operculata* host-pathogen association, a novel model in glycobiology and applied phycopathology. J Appl Phycol 13:185-193.

Box A, Martin D, Deudero S. (2010) Changes in seagrass polychaete assemblages after invasion by *Caulerpa racemosa* var. *cylindracea* (Chlorophyta: *Caulerpa*les): community structure, trophic guilds and taxonomic distinctness. Sci Mar 74:318–329.

Buia MC, Gambi MC, Terlizzi A, Mazzella L (2001) Colonization of *Caulerpa racemosa* along the southern Italian coasts: I. Distribution, phenological variability and ecological role. In: Gravez V, Ruitton S, Boudouresque CF, Le Direach L, Meinesz A, Scabbia G, Verlaque M (Eds.), Fourth International Workshop on *Caulerpa taxifolia*. GIS Posidonie Publisher, Marseille, France, pp. 352–360.

Bulleri F, Benedetti-Cecchi L (2008) Facilitation of the introduced green alga, *Caulerpa racemosa*, by resident algal turfs: experimental evaluation of underlying mechanisms. Mar Ecol-Prog Ser 364:77–86.

Bulleri F, Balata D, Bertocci I, Tamburello L, Benedetti-Cecchi L (2010) The seaweed *Caulerpa racemosa* on Mediterranean rocky reefs: from passenger to driver of ecological change. Ecology 91(8):2205–2212.

Burke C, Thomas T, Lewis M, Steinberg P, Kjelleberg S (2011a) Composition, uniqueness and variability of the epiphytic bacterial community of the green alga *Ulva australis*. ISME J 5:590–600.

Burke C, Steinberg P, Rusch D, Kjelleberg S, Thomas T (2011b) Bacterial community assembly based on functional genes rather than species. Proc Natl Acad Sci USA 108:14288–14293.

Byers JE, Wright JT, Gribben PE (2010) Variable direct and indirect effects of a habitat modifying invasive species on mortality of native fauna. Ecology 91:1787-1798. doi:10.1890/09-0712.1. PubMed: 20583719.

Callaway RM, Ridenour WM (2004) Novel weapons: invasive success and the evolution of increased competitive ability. Front Ecol Environ 2 (8):436–443.

Cao RS, Alaerts GJ (1995) Influence of reactor type and shear stress on aerobic biofilm morphology, population and kinetics. Water Res 29:107–118.

Capiomont A, Breugnot E, Den Haan M, Meinesz A (2005) Phenology of a deep-water population of *Caulerpa racemosa* var. *cylindracea* in the northwestern Mediterranean Sea. Bot Mar 48:80–83.

Carman KR, Dobbs FC (1997) Epibiotic microorganisms on copepods and other aquatic crustaceans. Micros Res Tech 37:116–135.

Carruthers TJB, Walker DI, Huisman JM (1993) Culture studies on two morphological types of *Caulerpa* (Chlorophyta) from Perth, Western Australia, with a description of a new species. Bot Mar 36:589–596.

Carvalho N, Liddle L, Caye G, Meinesz A (1998) Current knowledge on the biological cycle of the genus *Caulerpa* and karyological studies on *Caulerpa taxifolia*. In: Boudouresque C-F, Gravez V, Meinesz A, Palluy F (eds) 3rd International workshop on *Caulerpa taxifolia*, Marseille, 19–20 September 1997. GIS Posidonie, Marseille, pp 127–132.

Cavas L, Yurdakoc K (2005) A comparative study: Assessment of the antioxidant system in the invasive green alga *Caulerpa racemosa* and some macrophytes from the Mediterranean. J Exp Mar Biol Ecol 321:35–41.

Cavas L, Baskin Y, Yurdakoc K, Olgun N (2006) Antiproliferative and newly attributed apoptotic activities from an invasive marine alga: *Caulerpa racemosa* var. *cylindracea*. J Exp Mar Biol Ecol 339:11–119.

Cebrian E, Linares C, Marchal C, Garrabou J (2012) Exploring the effects of invasive algae on the persistence of gorgonian populations. Biol Invasions 14:2647–2656.

Ceccherelli G, Piazzi L (1999) The effect of *Posidonia oceanica* orientation of patch margin and density of shoots on the introduced alga *Caulerpa racemosa*. Biol Mar Medit 6 (1):365–367.

Ceccherelli G, Piazzi L, Cinelli F (2000) Response of the nonindigenous *Caulerpa racemosa* (Forsskal) J. Agardh to the native seagrass *Posidonia oceanica* (L.) Delile: effect of density of shoots and orientation of edges of meadows. J Exp Mar Biol Ecol 243:227–240.

Ceccherelli G, Piazzi L (2001) Dispersal of *Caulerpa racemosa* fragments in the Mediterranean: lack of detachment time effect on establishement. Bot Mar 44:209–213.

Ceccherelli G, Campo D (2002) Different effects of *Caulerpa racemosa* on two co-occurring seagrasses in the Mediterranean. Bot Mar 45:71–76.

Cerrano C, Puce S, Chiantore M, Bavestrello G, Cattaneo-Vietti, R (2001) The influence of the epizoic hydroid *Hydractinia angusta* on the recruitment of the Antarctic scallop *Adamussium colbecki*. Polar Biol 24(8):577–581.

Chan ECS, Mcmanus EA (1969) Distribution characterization and nutrition of marine microorganisms from algae *Polysiphonia Lanosa* and *Ascophyllum Nodosum*. Can J Microbiol 15:409–420.

Chiavelli DA, Mills EL, Threlkeld ST (1993) Host preference, seasonality, and community interactions of zooplankton epibionts. Limnol and Oceanogr 38(3):574–583.

Childress JJ, Fisher CR (1992) The biology of hydrothermal vent animals: physiology, biochemistry, and autotrophic symbioses. Oceanogr Mar Biol 30:337-441.

Claudet J, Fraschetti F (2010) Human-driven impacts on marine habitats: A regional meta-analysis in the Mediterranean Sea. Biol Conserv 143:2195-2206.

Coll M, Piroddi C, Steenbeek J, Kaschner K, Lasram FBR, Aguzzi J, Voultsiadou E (2010) The biodiversity of the Mediterranean Sea: estimates, patterns, and threats. PloS one 5(8):e11842.

Cooksey KE, Wigglesworth-Cooksey B (1995) Adhesion of bacteria and diatoms to surfaces in the sea: a review. Aquat Microb Ecol 9:87–96.

Correa JA (1997) Infectious diseases of marine algae: current knowledge and approaches. In: Round FE, Chapman DJ (eds) Progress in phycological research. Biopress, Bristol, UK, pp 149–180.

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. Annu Rev Microb 41: 435–464.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. Annu Rev Microb 49:711–745.

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438:90–93.

Croft MT, Warren MJ, Smith AG (2006) Algae need their vitamins. Eukaryot Cell 5:1175–1183.

Dahms H-U, Qian P-Y (2005) Exposure of biofilms to meiofaunal copepods affects the larval settlement of *Hydroides elegans* (Polychaeta). Mar Ecol-Prog Ser 297:203–214.

Dang H, Lovell CR (2000) Bacterial primary colonization and early succession on surfaces in marine waters as analyzed by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. Appl Environ Microb 66:467–475.

Davis AR, White GA (1994) Epibiosis in a guild of sessile subtidal invertebrates in southeastern Australia: a quantitative survey. J Exp Mar Biol Ecol 177(1):1–14.

Dobretsov SV, Qian PY (2002) Effect of bacteria associated with the green alga Ulva reticulata on marine micro- and macrofouling. Biofouling 18:217–228.

Dobretsov S, Qian PY (2004) The role of epibotic bacteria from the surface of the soft coral *Dendronephthya* sp. in the inhibition of larval settlement. J Exp Mar Biol Ecol 299:35–50.

Dobretsov S, Qian P-Y (2006) Facilitation and inhibition of larval attachment of the bryozoan *Bugula neritina* in association with mono-species and multi-species biofilms. J Exp Mar Biol Ecol 333:263–274.

Dobretsov S, Dahms H-U, Qian P-Y (2006) Inhibition of biofouling by marine microorganisms and their metabolites. Biofouling 22:43–54.

Dobretsov S, Dahms H-U, Huang YL, Wahl M, Qian P-Y (2007) The effect of quorum sensing blockers on the formation of marine microbial communities and larval attachment. FEMS Microbiol Ecol 60:177–188.

Dobretsov S (2009) Marine Biofims. In: Biofouling., ed. by Dürr, S. and Thomason, J. Wiley-Blackwell, Weinheim, pp. 100-108. ISBN 978-1-4051-6926-4.

Dougherty JR, Russell MP (2005) The association between the coquina clam *Donax fossor* Say and its epibiotic hydroid *Lovenella gracilis* Clarke. J Shellfish Res 24 (1):35–46.

Dreher TW, Grant BR, Wetherbee R (1978) The wound response in the siphonous *Caulerpa* simpliciuscula C. Ag.: Fine structure and cytology. Protoplasma 96(1–2):189–203.

Dumay O, Pergent G, Pergent-Martini C, Amade P. (2002) Variations in caulerpenyne contents in *Caulerpa taxifolia* and *Caulerpa racemosa*. J Chem Ecol 28(2):343–352.

Egan S, Thomas T, Holmstrom C, Kjelleberg S (2000) Phylogenetic relationship and antifouling activity of bacterial epiphytes from the marine alga Ulva lactuca. Environ Microbiol 2:343–347.

Egan S, Holmstrom C, Kjelleberg S (2001) Pseudoalteromonas ulvae sp nov., a bacterium with antifouling activities isolated from the surface of a marine alga. Int J Syst Evol Microbiol 51:1499–1504.

Egan S, Thomas T, Kjelleberg S (2008) Unlocking the diversity and biotechnological potential of marine surface associated microbial communities. Curr Opin Microbiol 11:219–225.

Eilers H, Pernthaler J, Gloeckner FO, Amann R (2000) Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl Environ Microbiol 66:3044–3051.

Enticknap JJ, Kelly Shanks M, Peraud O, Hill RT (2006) Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. Appl Environ Microbiol 72:4105-4119.

Eriksson BK, Johansson G (2003) Sedimentation reduces recruitment success of Fucus vesciculosus (Phaeophyceae) in the Baltic Sea. Eur J Phycol 38:217-222.

Facon B, Pointier JP, Jarne P, Sarda V, David P (2008) High genetic variance in life-history strategies within invasive populations by way of multiple introductions. Curr Biol 18:363–367. doi:10.1016/j.cub. 2008.01.063. PubMed: 18334202.

Fama` P, Olsen JL, Stam WT, Procaccini G (2000) High levels of intraand inter-individual polymorphism in the rDNS ITS1 of *Caulerpa racemosa* (Chlorophyta). Eur J Phycol 35:349–356.

Felline S, Caricato R, Cutignano A, Gorbi S, Lionetto MG, Mollo E, Regoli F, Terlizzi A (2012) Subtle Effects of Biological Invasions: Cellular and Physiological Responses of Fish Eating the Exotic Pest *Caulerpa racemosa*. PLoSONE 7(6):e38763. doi:10.1371/journal.pone.0038763

Fenchel T (1986) The ecology of heterotrophic microflagellates. Adv Microb Ecol 9:57–97.

Fernandez L, Parapar J, Gonzalez-Gurriaran E, Muino R (1998) Epibiosis and ornamental cover patterns of the spider crab *Maja squinado* on the Galician coast, northwestern Spain: Influence of behavioral and ecological characteristics of the host. J Crustacean Biol 18(4):728–737.

FlemminG H-C, Wingender J, Griebe T, Mayer C (2001) Physico-chemical properties of biofilms. In: Biofilms: Recent Advances in their Study and Control (ed. L.V. Evans), pp. 19–34. Harwood Academic Publishers, Amsterdam.

Fletcher M, Loeb G (1979) Influence of substratum characteristics on the attachment of marine pseudomonas to solid surfaces. Appl Environ Microb 37:67–72.

Forster RM, Creach V, Sabbe K, Vyverman W, and Stal LJ (2006) Biodiversity-ecosystem function relationship in microphytobenthic diatoms of the Westerschelde estuary. Mar Ecol-Prog Ser 311:191–201.

Friedrich MW (2012). Bacterial communities on macroalgae, in: Wiencke, C. et al. (Ed.) (2012). Seaweed biology: Novel insights into ecophysiology, ecology and utilization. Ecological Studies, 219: pp. 189-201 In: Wiencke C; Bischof K (Ed.) (2012). Seaweed biology: Novel insights into ecophysiology, ecology and utilization. Ecological Studies, 219. Springer-Verlag: Berlin, Heidelberg. ISBN 978-3-642-28450-2. XIII, 510 pp.

Fuhrman JA, Campbell L (1998) Microbial microdiversity. Nature 393:410–411.

Goecke F, Labes A, Wiese J, Imhoff JF (2010) Chemical interactions between marine macroalgae and bacteria. Mar Ecol-Prog Ser 409:267–299.

Green J, Bohannan BJM (2006) Spatial scaling of microbial biodiversity. Trends Ecol Evol 21:501–507.

Harder T, Lau SCK, Dobretsov S, Fang TK Qian PY (2003) A distinctive epibiotic bacterial community on the soft coral *Dendronephthya* sp. and antibacterial activity of coral tissue extracts suggest a chemical mechanism against bacterial epibiosis. FEMS Microbiol Ecol 43(3):337–347.

Hellio C, De La Broise D, Dufossé L, Le Gal Y, Bourgougnon N (2001) Inhibition of marine bacteria by extracts of macroalgae: Potential use for environmentally friendly antifouling paints. Mar Environ Res 52:231-247.

Hendricks IE, Bouma TJ, Morris EP, Duarte CM (2010) Effects of seagrasses and algae of the *Caulerpa* family on hydrodynamics and particle-trapping rates. Mar Biol 157:73–481.

Hiddink JG, Ben Rais Lasram F, Cantrill J, Davies AJ (2012) Keeping pace with climate change: what can we learn from the spread of Lessepsian migrants? Glob Change Biol 18(7):2161–2172.doi:10.1111/j.1365-2486.2012.02698.x

Holmer M, Marbà N, Lamote M, Duarte CM (2009) Deterioration of Sediment Quality in Seagrass Meadows (*Posidonia oceanica*) Invaded by Macroalgae (*Caulerpa* sp.). Estuar Coast 32:456-466.

Holmstrom C, Kjelleberg S (1999) Marine *Pseudoalteromonas* species are associated with higher organisms and produce active extracellular compounds. FEMS Microbiol Ecol 30:285–293.

Huggett MJ, Crocetti GR, Kjelleberg S, Steinberg PD (2008) Recruitment of the sea urchin Heliocidaris erythrogramma and the distribution and abundance of inducing bacteria in the field. Aquat Microb Ecol 53:161–171.

Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJM (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol 67:4399–4406.

Irving AD, Connell SD (2002a) Sedimentation and light penetration interact to maintain heterogeneity of subtidal habitats: algal versus invertebrate dominated assemblages. Mar Ecol-Prog Ser 245:83-91.

Irving AD, Connell SD (2002b) Interactive effects of sedimentation and microtopography on the abundance of subtidal turf-forming algae. Phycologia 41:517-522.

Jensen PR, Kauffman CA, Fenical W (1996) High recovery of culturable bacteria from the surfaces of marine algae. Mar Biol 126:1–7.

Johnson CR, Muir DG, Reysenbach AL (1991) Characteristic bacteria associated with surfaces of coralline algae: a hypothesis for bacterial induction of marine invertebrate larvae. Mar Ecol Prog Ser 74:281–294.

Joint I, Tait K, Callow ME, Callow JA, Milton D, Williams P, Camara M (2002) Cell-to-cell communication across the prokaryote-eukaryote boundary. Science 298:1207.

Joint I, Tait K, Wheeler G (2007) Cross-kingdom signalling: exploitation of bacterial quorum sensing molecules by the green seaweed *Ulva*. Philos Trans R Soc Lond B Biol Sci 362:1223–1233.

Jones PR, Cottrell MT, Kirchman DL, Dexter SC (2007) Bacterial community structure of biofilms on artificial surfaces in an estuary. Microb Ecol 53:153–162.

Joubert LM, Wolfaardt BA (2006) Microbial exopolymers link predator and prey in a model yeast biofilm system. Microb Ecol 52:187–197.

Jung V, Thibault T, Meinesz A, Pohnert G (2002) Comparison of the wound-activated transformation of Caulerpenyne by invasive and noninvasive *Caulerpa* species of the Mediterranean Sea. J Chem Ecol 28(10):2091–2105.

Keane RM, Crawley MJ (2002) Exotic plant invasions and the enemy release hypothesis. Trends Ecol Evol 17:164–170.

Keshtacherliebson E, Hadar Y, Chen Y (1995) Oligotrophic bacteria enhance algal growth under iron-deficient conditions. Appl Environ Microbiol 61:2439–2441.

Kiørboe T, Tang K, Grossart HP, Ploug H (2003) Dynamics of microbial communities on marine snow aggregates: colonization, growth, detachment, and grazing mortality of attached bacteria. Appl Environ Microbiol 69:3036–3047.

Klein J, Verlaque M (2008) The *Caulerpa racemosa* invasion: A critical review. Mar Pollut Bull 56:205–225. doi:10.1016/j.marpolbul. 2007.09.043. PubMed: 18061625.

Klein J, Verlaque M (2009) Macroalgal assemblages of disturbed coastal detritic bottoms subject to invasive species. Est Coast Sh Sci 82:461–468.

Kong MK, Chan KY (1979) A study on the bacterial flora isolated from marine algae. Bot Mar 22:83-97.

Lachnit T, Meske D, Wahl M, Harder T, Schmitz R (2011) Epibacterial community patterns on marine macroalgae are host-specific but temporally variable. Environ Microbiol 13:655–665.

Lam C, Stang A, Harder T (2008) Planktonic bacteria and fungi are selectively eliminated by exposure to marine macroalgae in close proximity. FEMS Microbiol Ecol 63:283-291.

Lane AL, Kubanek J (2008) Secondary metabolite defenses against pathogens and biofoulers. pp. 229-243. In: Amsler CD (ed.) (2008) Algal chemical ecology, 313 pp. Springer-Verlag Berlin Heidelberg.

Largo DB, Fukami K, Nishijima T (1995) Occasional pathogenic bacteria promoting ice-ice disease in the carrageenan producing red algae Kappaphycus alvarezii and Eucheuma denticulatum (Solieriaceae, Gigartinales, Rhodophyta). J Appl Phycol 7:545–554.

Largo DB, Fukami K, Adachi M, Nishijima T (1997) Direct enumeration of bacteria from macroalgae by epifluorescence microscopy as applied to the fleshy red algae Kappaphycus alvarezii and Gracilaria spp. (Rhodophyta). J Phycol 33:554–557.

Largo DB, Fukami K, Nishijima T (1999) Time-dependent attachment mechanism of bacterial pathogen during ice-ice infection in Kappaphycus alvarezii (Gigartinales, Rhodophyta). J Appl Phycol 11:129–136.

Lau SCK, Thiyagarajan V, Cheung SCK, Qian P-Y (2005) Roles of bacterial community composition in biofilms as a mediator for larval settlement of three marine invertebrates. Aquat Microb Ecol 38:41–51.

Laudien J, Wahl M (1999) Indirect effects of epibiosis on host mortality: starfish predation on differently fouled mussels. Marine Ecology-Pubblicazioni Della Stazione Zoologica Di Napoli I **20** (1):35–47.

Laudien J, Wahl M (2004) Associational resistance of fouled blue mussels (*Mytilus edulis*) against starfish (*Asterias rubens*) predation: relative importance of structural and chemical properties of the epibionts. Helgoland Mar Res 58 (3):162–167.

Lejeusne C, Chevaldonné P, Pergent-Martini C, Boudouresque CF, Pérez T (2010) Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. Trends Ecol Evol 25:250-260.

Lema KA, Willis BL, Bourne DG (2012) Corals form characteristic associations with symbiotic nitrogen-fixing bacteria. Appl Environ Microbiol 78 (9):3136–3144.

Lewandowski Z (2000) Structure and function of biofilms. In: Biofilms: Recent Advances in their Study and Control (ed. L.V. Evans), pp. 1–17. Harwood Academic Publishers, Amsterdam.

Lewis TE, Garland CD, Mcmeekin TA (1985) The bacterial biota on crustose (nonarticulated) coralline algae from tasmanian waters. Microb Ecol 11:221–230.

Li M, Qian PY (2005) Antagonistic antimicrobial activity of marine fungi and bacteria isolated from marine biofilm and seawaters of Hong Kong. Aquat Microb Ecol 38:231–238.

Liesack W, Janssen PH, Rainey FA, Ward-Rainey NL, Stackebrandt E (1997) Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques. In: van Elsas JD, Trevors JT, Wellington EMH (eds) Modern soil microbiology. Marcel Dekker, New York, pp 375–439.

Lipkin Y (1975) Ecological distribution of *Caulerpa* in the Red Sea. J mar biol Assoc India 15:160-167.

Liu M, Dong Y, Zhao Y, Zhang GT, Zhang WC, Xiao T (2011) Structures of bacterial communities on the surface of *Ulva prolifera* and in seawaters in an *Ulva* blooming region in Jiaozhou Bay, China. World J Microbiol Biotechnol 27:1703–1712.

Liu WT, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522.

Lodge DM, Williams S, MacIsaac HJ, Hayes KR, Leung B, Reichard S, Mack RN, Moyle PB, Smith M, Andow DA, Carlton JT, McMichael A (2006) Biological invasions: Recommendations for US policy and management. Ecol Appl 16:2035–2054.

Lorenti M, Gambi MC, Guglielmo R, Patti FP, Scipione MB, Zupo V, Buia MC (2011) Soft bottom macrofaunal assemblages in the Gulf of Salerno, Tyrrhenian Sea, Italy, affected by the invasive seaweed *Caulerpa racemosa* var. *cylindracea*. Mar Ecol 32(3):320-334.

Lorenti M, Gambi MC, Guglielmo R, Patti FP, Scipione MB, Zupo V, Buia MC (2009) Agents of change in benthic marine assemblages: the effects of the invasive seaweed *Caulerpa racemosa* var. *cylindracea* on soft-bottom macrofauna. ASLO Aquatic Science meeting 2009, 25–30 January 2009, Nice: Abstract 158.

Maki JS (2002) Biofouling in the marine environment. In: Encyclopedia of Environmental Microbiology (ed. G. Bitton), pp. 610–619. John Wiley and Sons, New York.

Manning LM, Lindquist N (2003) Helpful habitant or pernicious passenger: interactions between an infaunal bivalve, an epifaunal hydroid and three potential predators. Oecologia 134(3):415–422.

Marin A, Belluga MDL (2005) Sponge coating decreases predation on the bivalve *Arca noae*. J Mollus Stud 71:1–6.

Marsh TL (2005) Culture-independent microbial community analysis with terminal restriction fragment length polymorphism. Environ Microbiol 397:308–329.

Marshall KC, Stout R, Mitchell R (1971) Mechanism of the initial events in the sorption of marine bacteria to surfaces. J Genet Microbiol 68:337–348.

Marshall K, Joint I, Callow ME, Callow JA (2006) Effect of marine bacterial isolates on the growth and morphology of axenic plantlets of the green alga *Ulva linza*. Microb Ecol 52:302–310.

Matijevic S, Bogner M, Bojanić N, Žuljević A, Despalatović M, Antolić B, Nikolić V, Bilić J (2013) Biogeochemical characteristics of sediments under the canopy of invasive alga *Caulerpa racemosa* var. *cylindracea* (Peljesac Peninsula, Adriatic Sea). Fresen Environ Bull 22:3030-3040.

Matsuo Y, Suzuki M, Kasai H, Shizuri Y, Harayama S (2003) Isolation and phylogenetic characterization of bacteria capable of inducing differentiation in the green alga Monostroma oxyspermum. Environ Microbiol 5:25–35.

Maugeri TL, Carbone M, Fera MT, Irrera GP, Gugliandolo C (2004) Distribution of potentially pathogenic bacteria as free living and plankton associated in a marine coastal zone. J Appl Microbiol 97:354–361.

Maybruck BT, Rogerson A (2004) Protozoan on the prop roots of the red mangrove tree, *Rhizophora mangle*. Protistology 3:265–272.

McEldowney S, Fletcher M (1986) Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces. J Dent Microb 132:513– 523.

McKinnon JG, Gribben PE, Davis AR, Jolley DF, Wright JT (2009) Differences in soft-sediment macrobenthic assemblages invaded by *Caulerpa taxifolia* compared to uninvaded habitats. Mar Ecol Prog Ser 380:59–71.

Meinesz A, Belsher T, Thibaut T, Antolic B, Ben Mustapha K, Boudouresque C-F, Chiaverini D, Cinelli F, Cottalorda J-M, Djellouli A, El Abed A, Orestano C, Grau AM, Ivesa L, Jaklin A, Langar H, Massuti-Pascual E, Peirano A, Tunesi L, de Vaugelas J, Zavodnik N, Zuljevic A (2001) The introduced green alga *Caulerpa taxifolia* continues to spread in the Mediterranean. Biol Invasions 3:201–210.

Meusnier I, Olsen JL, Stam WT, Destombe C, Valero M (2001) Phylogenetic analyses of *Caulerpa taxifolia* (Chlorophyta) and of its associated bacterial microflora provide clues to the origin to the Mediterranean introduction. Mol Ecol 10:931–946. doi:10.1046/j. 1365-294X.2001.01245.x. PubMed: 11348502.

Moss JA, Nocker A, Lepo JE Snyder RA (2006) Stability and change in estuarine biofilm bacterial community diversity. Appl Environ Microbiol 72:5679–5688.

Muyzer G, Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700.

Nakanishi K, Nishijima M, Nishimura M, Kuwano K, Saga N (1996) Bacteria that induce morphogenesis in Ulva pertusa (chlorophyta) grown under axenic conditions. J Phycol 32:479–482.

Nealson HK, Haygood GM, Tebo MB, Roman M, Miller E, McCosker EJ (1984) Contribution by symbiotically luminous fishes to the occurrence and bioluminescence of luminous bacteria in seawater. Microb Ecol 10:69–77.

Neira C, Levin LA, Grosholz ED (2005) Benthic macrofaunal communities of three sites in San Francisco Bay invaded by hybrid Spartina, with comparison to uninvaded habitats. Mar Ecol-Prog Ser 292:111–126.

Neira C, Grosholz ED, Levin LA, Blake R (2006) Mechanisms generating modification of benthos following tidal flat invasion by a Spartina hybrid. Ecol Appl 16:1391–1404.

Nishiguchi MK, Ruby EG, McFall-Ngai MJ (1998) Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid–Vibrio symbioses. Appl Environ Microbiol 64:3209–3213.

Nizamuddin M (1991) The Green Marine Algae of Libya. Elga Publisher, Bern.

Nocker A, Lepo JE, Snyder RA (2004) Influence of an oyster reed on development of the microbial heterotrophic community on an estuare biofilm. Appl Envirol Microbiol 70:6834–6845.

O'Toole G, Kaplan HB, Kolter R (2000) Biofilm formation as microbial development. Annu Rev Microbiol 54:49–79.

Olafsen JA (2001) Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 200:223–247.

Olson JB, Kellogg CA (2010) Microbial ecology of corals, sponges, and algae in mesophotic coral environments. FEMS Microbiol Ecol 73:17–30.

Pacciardi L, De Biasi AM, Piazzi L (2011) Effects of *Caulerpa racemosa* invasion on soft-bottom assemblages in the Western Mediterranean Sea Biol Invasions 13(12):2677-2690. Doi 10.1007/s10530-011-9938-5.

Pace NR, Stahl DA, Lane DJ, Olsen GJ (1986) The analysis of natural microbial populations by ribosomal RNA sequences. Adv Microb Ecol 9:1–55.

Panayotidis P, Montesanto B (1994) *Caulerpa racemosa* (Chlorophyta) on the Greek coasts. Cryptogamie Algol 15:159–161.

Panayotidis P, Zuljevic A (2001) Sexual reproduction of the invasive green alga *Caulerpa racemosa* var. occidentalis in the Mediterranean sea. Oceanol Acta 24:199–203.

Pandolfo A, Chemello R (1995) Prime note sulla malacofauna associata a *Caulerpa racemosa* nella Baia di Santa Panagia (Sicilia Orientale). Biol Mar Medit 2(2):649–651.

Patel P, Callow ME, Joint I, Callow JA (2003) Specificity in the settlement–modifying response of bacterial biofilms towards zoospores of the marine alga Enteromorpha. Environ Microbiol 5:338–349.

Penesyan A, Kjelleberg S, Egan S (2010) Development of Novel Drugs from Marine Surface Associated Microorganisms. Mar Drugs 8(3):438–459.

Pernthaler J, Amann R (2005) Fate of heterotrophic microbes in pelagic habitats: focus on populations. Microbiol Mol Biol Rev 69:440–461.

Piazzi L, Balestri E, Magri M, Cinelli F (1997a) Expansion de l'algue tropicale *Caulerpa racemosa* (Forsskal) J. Agardh (Bryopsidophyceae, Chlorophyta) le long de la coˆ te toscane (Italie). Cryptogamie Algol 18:343–350.

Piazzi L, Acunto S, Magri M, Rindi F, Balestri E (1997b) Osservazioni preliminari sull'espansione di *Caulerpa racemosa* (Forsskal) J. Agardh sulle Secche della Meloria (Livorno, Italia). Biol Mar Medit 4(1):426–428.

Piazzi L, Cinelli F (1999) Developpement et dynamique saisonniere d'un peuplement mediterraneen de l'algue tropicale *Caulerpa racemosa* (Forskal) J. Agardh. Cryptogamie Algol 20:295–300.

Piazzi L, Ceccherelli, G, Cinelli F (2001) Threat to macroalgal diversity: effects of the introduced green alga *Caulerpa racemosa* in the Mediterranean. Mar Ecol-Prog Ser 210:149–159.

Piazzi L, Balata D, Ceccherelli G, Cinelli F (2005a) Interactive effect of sedimentation and *Caulerpa racemosa* var. *cylindracea* invasion on macroalgal assemblages in the Mediterranean Sea. Estuar. Coast Shelf Sci 64:467-474.

Piazzi L, Meinesz A, Verlaque M, Akcali B, Antolic´ B, Argyrou M, Balata D, Ballesteros E, Calvo S, Cinelli F, Cirik S, Cossu A, D'Archino R, Dellouli AS, Javel F, Lanfranco OE, Mifsud C, Pala D, Panayotidis P, Peirano A, Pergent G, Petrocelli A, Ruitton S, Z ˇ uljevic´ A, Ceccherelli G (2005b) Invasion of *Caulerpa racemosa* var. *cylindracea* (*Caulerpa*les, Chlorophyta) in the Mediterranean Sea: an assessment of the spread. Cryptogamie Algol 26:189–202.

Piazzi L, Balata D, Foresi L, Cristaudo C, Cinelli F (2007) Sediment as a constituent of Mediterranean benthic communities dominated by *Caulerpa racemosa* var. *cylindracea*. Sci Mar 71(1):129–135.

Piazzi L, Balata D (2007) The spread of *Caulerpa racemosa* var. *cylindracea* in the Mediterranean Sea: an example of how biological invasion can influence beta diversity. Mar Env Res 65:50–61.

Piazzi L, Balata D (2009) Invasion of alien macroalgae in different Mediterranean habitats. Biol Inv 11:193–204.

Provasoli L, Pintner IJ (1980) Bacteria induced polymorphism in an axenic laboratory strain of Ulva lactuca (Chlorophyceae). J Phycol 16:196–201.

Pujalte MJ, Ortigosa M, Macian MC, Garay E (1999) Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. Int Microbiol 2:259–266.

Qian P-Y, Lau SCK, Dahms H-U, Dobretsov S, Harder T (2007) Marine biofilms as mediators of colonization by marine macroorganisms: implications for antifouling, aquaculture, and conservation management. Mar Biotechnol 9:399–410.

Raghukumar C, Nagarkar S, Raghukumar S (1992) Association of thraustochytrids and fungi with living marine algae. Mycol Res 96:542–546.

Railkin AI (2004) *Marine Biofouling: Colonization Processes and Defenses*. CRC Press, Boca Raton.

Rao D, Webb JS, Kjelleberg S (2005) Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicate*. Appl Environ Microbiol 71:1729–1736.

Rappe MS, Giovannoni SJ (2003) The uncultured microbial majority. Annu Rev Microbiol 57:369–394.

Renoncourt L, Meinesz A (2002) Formation of propagules on an invasive strain of *Caulerpa racemosa* (Chlorophyta) in the Mediterranean Sea (Research Note). Phycologia 41:533–535.

Ricciardi A, Cohen J (2007) The invasiveness of an introduced species does not predict its impact. Biol Invasions 9:309–315.

Rosowski JR (1992) Specificity of bacterial attachment sites on the filamentous diatom Navicula confervacea (Bacillariophyceae). Can J Microbiol 38:676–686.

Rossello-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25:39–67.

Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environment. Microbiol Rev 51:365–379.

Ruitton S, Verlaque M, Boudouresque CF (2005) Seasonal changes of the introduced *Caulerpa racemosa* var. *cylindracea* (*Caulerpa*les, Chlorophyta) at the northwest limit of its Mediterranean distribution. Aquat Bot 82:55–70.

Ruitton S, Verlaque M, Aubin, G, Boudouresque CF (2006) Grazing on *Caulerpa racemosa* var. *cylindracea* (*Caulerpa*les, Chlorophyta) in the Mediterranean Sea by herbivorous fish and sea urchins. Vie et Milieu 56 (1):33–41.

Sale PF (1979) Recruitment, loss and coexistence in a guild of territorial coral-reef fishes. Oecologia 42:159–177.

Santavy DL, Colwell RR (1990) Comparison of bacterial communities associated with the Caribbean sclerosponge Ceratoporella nicholsoni and ambient seawater. Mar Ecol-Prog Ser 67:73–82.

Schaffelke B, Smith JE, Hewitt CL (2006) Introduced macroalgae—a growing concern. J Appl Phycol 18:529–41.

Serio D, Pizzuto F (1998) Studio di un prato a *Caulerpa racemosa* (Forsskal) J. Agardh (*Caulerpa*les, Chlorophyta) di Brucoli (SR) con osservazioni in coltura della specie. Bollettino dell'Accademia Gioenia di Scienze Naturali Catania 31 (354), 201–209.

Smith CM, Walters LJ (1999) Fragmentation as a strategy for *Caulerpa* species: Fates of fragments and implications for management of an invasive weed. Mar Ecol 20:307–19.

Stabili L, Gravili C, Piraino S, Boero F, Alifano P (2006) *Vibrio harveyi* associated with *Aglaophenia octodonta* (Hydrozoa, Cnidaria). Microb Ecol 52:603–608.

Stabili L, Gravili C, Tredici S, Piraino S, Tala` A, Boero F, Alifano P (2008) Epibiotic Vibrio luminous bacteria isolated from some Hydrozoa and Bryozoa species. Microb Ecol 56:625–636.

Staufenberger T, Thiel V, Wiese J, Imhoff JF (2008) Phylogenetic analysis of bacteria associated with Laminaria saccharina. FEMS Microbiol Ecol 64:65–77.

Steinberg PD, de Nys R (2002) Chemical mediation of colonization of seaweed surfaces. J Phycol 38:621–629.

Stevenson MD, Rossmo DK, Knell RJ, Le Comber SC (2012) Geographic profiling as a novel spatial tool for targeting the control of invasive species. Ecography 35:1–12.

Stewart FJ, Cavanaugh CM (2006) Bacterial endosymbiosis in Solemya (Mollusca: Bivalvia)—model systems for studies of symbiont-host adaptation. Antonie van Leeuwenhoeck 90:343–360.

Stoodley P, Sauer K, Davies DG, Costerton JW (2002) Biofilms as complex differentiated communities. Ann Rev Microbiol 56:187–209.

Strayer DL, Eviner VT, Jeschke JM, Pace ML (2006) Understanding the long-term effects of species invasions. Trends Ecol Evol 21:645–651.

Streftaris N, Zenetos A (2006) Alien Marine Species in the Mediterranean – the 100 'Worst Invasives' and their Impact. Mediterr Mar Sci 7:87-118.

Tait K, Joint I, Daykin M, Milton DL, Williams P, Camara M (2005) Disruption of quorum sensing in seawater abolishes attraction of zoospores of the green alga *Ulva* to bacterial biofilms. Environ Microbiol 7:229–240.

Thevanathan R, Nirmala N, Manoharan A, Gangadharan A, Rajarajan R, Dhamotharan R, Selvaraj S (2000) On the occurrence of nitrogen fixing bacteria as epibacterial flora of some marine green algae. Seaweed Res Utiln 22:189–197.

Thomas D (2002) Seaweeds. The Natural History Museum, London. 96 pp.

Thompson, RC, Moschella PS, Jenkins SR, Norton TA, Hawkins SJ (2002) Differences in photosynthetic marine biofilms between sheltered and moderately exposed rocky shores. Mar Ecol-Prog Ser 296:53–63.

Thomsen MS, Wenberg T, Tuya F, Silliman BR (2009) Evidence for impacts of nonindigenous macroalgae: a meta-analysis of experimental field studies. J Phycol 45:812–819.

Thornton DCO, Dong LF, Underwood GJC, Nedwell DB (2002) Factors affecting microphytobenthic biomass species composition and production in the Colne Estuary (UK). Aquat Microb Ecol 27:285–300.

Thresher RE (2000) Key threats from marine bioinvasions: a review of current and future issues. p. 24-34. In: Marine bioinvasions: Proceedings of a conference January 24-27, 1999. MIT Sea Grant College Program. Pederson, J. (Ed.).

Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM (2003) Introduced species and their missing parasites. Nature 421:628–630.

Tsirika A, Haritonidis S (2005) A survey of the benthic flora in the National Park of Zakynthos (Greece). Bot Mar 48:38–45.

Tujula NA, Crocetti GR, Burke C, Thomas T, Holmstrom C, Kjelleberg S (2010) Variability and abundance of the epiphytic bacterial community associated with a green marine Ulvacean alga. ISME J 4:301–311.

Turnes G, Turnes MS, Fenical W (1992) Embryos of *Homarus americanus* are protected by epibiotic bacteria. Biol Bull 182 (1):105–108.

Underwood GJC (2005) Microalgal (Microphytobenthic) biofilms in shallow coastal waters: how important are species? *Proceedings California Academy of Sciences* 56**:**162–169.

UNEP-MAP-RAC/SPA, Marine Mediterranean Invasive Alien Species (MAMIAS), 2012: http://www.mamias.org

Vairappan CS, Suzuki M, Motomura T, Ichimura T (2001) Pathogenic bacteria associated with lesions and thallus bleaching symptoms in the Japanese kelp Laminaria religiosa Miyabe (Laminariales, Phaeophyceae). Hydrobiologia 445:183–191.

Vandevivere P, Kirchman DL (1993) Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl Environ Microbiol 59:3280–3286.

Vazquez-Luis M, Sanchez-Jerez P, Bayle-Sempere JT (2009) Comparison between amphipod assemblages associated with *Caulerpa racemosa* var. *cylindracea* and those of other Mediterranean habitats on soft substrates. Estuar Coast Shelf Sci 84:161–170.

Verlaque M, Durand C, Huisman JM, Boudouresque CF, Le Parco Y (2003) On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). Eur J Phycol 38:325–339.

Vignesh S, Raja A, Arthur James R (2011) Marine drugs: Implication and future studies. International J of Pharmacol 7:22–30.

Vitousek P, D'Antonio CM, Loope L, Westbrooks R (1996) Biological invasions as global environmental change. Am Sci 84:468–478.

Wahl M, Hay ME (1995) Associational resistance and shared doom: effects of epibiosis on herbivory. Oecologia 102:329–340.

Wahl M, Hay ME, Enderlein P (1997) Effects of epibiosis on consumer-prey interactions. Hydrobiologia 355:49–59.

Wahl M (1997) Living attached: aufwuchs, fouling, epibiosis. In: Fouling Organisms of the Indian Ocean: Biology and Control Technology (eds R. Nagabhushanam and M. Thompson), pp. 31–84. Oxford and IBH Publishing, New Delhi.

Wahl M (2008) Ecological lever and interface ecology: epibiosis modulates the interactions between host and environment. Biofouling 24:427–438.

Wahl M (2009) Epibiosis In: Biofouling., ed. by Dürr, S. and Thomason, J. Wiley-Blackwell, Weinheim, pp. 100-108. ISBN 978-1-4051-6926-4

Wallentinus I, Nyberg CD (2007) Introduced marine organisms as habitat modifiers. Mar Pollut Bull 55:323–332.

Walls JT, Ritz DA, Blackman MJ (1993) Fouling, surface bacteria and antibacterial agents of four bryozoan species found in Tasmania, Australia. Exp Mar Biol Ecol 169:1–13.

Wang G, Shuai L, Li Y, Lin W, Zhao XW, Duan DL (2008) Phylogenetic analysis of epiphytic marine bacteria on Hole-Rotten diseased sporophytes of Laminaria japonica. J Appl Phycol 20:403–409.

Webster NS, Smith LD, Heyward AJ, Watts JE, Webb RI, Blackall LL, Negri AP (2004) Metamorphosis of a scleractinian coral in response to microbial biofilms. Appl Environ Microbiol 70:1213–1221.

Weinbauer M (2004) Ecology of prokaryotic viruses. FEMS Microbiol Rev 28:127–181.

Weinberger F, Hoppe HG, Friedlander M (1997) Bacterial induction and inhibition of a fast necrotic response in Gracilaria conferta (Rhodophyta). J Appl Phycol 9:277-285.

Weinberger F, Beltran J, Correa JA, Lion U, Pohnert G, Kumar N, Steinberg P, Kloareg B, Potin F (2007) Spore release in Acrochaetium sp (Rhodophyta) is bacterially controlled. J Phycol 43:235–241.

Wieczorek SK, Todd CD (1998) Inhibition and facilitation of the settlement of epifaunal marine invertebrate larvae by microbial biofilm cues. Biofouling 12:81–93.

Wigglesworth-Cooksey B, Cooksey KE (2005) Use of fluorophore-conjugated lectins to study cell-cell interactions in model marine biofilms. Appl Environ Microbiol 71:428–435.

Wilcover DS, Rothstein D, Dubow J, Phillips A, Losos E (1998) Quantifying threats to imperilled species in the United States. Bioscience 48:607–615.

Williams SL, Smith JE (2007) A global review of the distribution, taxonomy andimpacts of introduced seaweeds. Annu Rev Ecol Syst 38:327–359.

Wright JT, Davis AR (2006) Demographic feedback between clonal growth and fragmentation in an invasive seaweed. Ecology 87, 1744–1754.

Zenetos A, Gofas S, Verlaque M, Çinar ME, Garcia Raso JE, Bianchi CN, Morri C, Azzurro E, Bilecenoglu M, Froglia C, Siokou I, Violanti D, Sfriso A, San Martin G, Giangrande A, Katagan T, Ballesteros E, Ramos-Espla AA, Mastrototaro F, Ocana O, Zingone A, Gambi MC, Streftaris N (2010) Alien species in the Mediterranean Sea by 2010. A contribution to the application of European Union's Marine Strategy Framework Directive (MSFD). Part I. Spatial distribution. Mediterr Mar Sci 11(2):318-493.

Zenetos A, Gofas S, Verlaque M, Çinar ME, Garcia Raso JE, Bianchi CN, Morri C, Azzurro E, Bilecenoglu M, Froglia C, Siokou I, Violanti D, Sfriso A, San Martin G, Giangrande A, Katagan T, Ballesteros E, Ramos-Espla AA, Mastrototaro F, Ocana O, Zingone A, Gambi MC, Streftaris N (2011) Alien species in the Mediterranean Sea by 2010. Errata to the Review Article (Medit. Mar. Sci. 11/2, 2010, 381-493). Mediterr Mar Sci 12(2): 509-514.

Zenetos A, Gofas S, Morri C, Rosso A, Violanti D, Garcia Raso JE, Cinar ME, Almogi-Labin A, Ates AS, Azzurro E, Ballesteros E, Bianchi CN, Bilecenoglu M, Gambi MC, Giangrande A, Gravili C, Hyams-Kaphzan O, Karachle PK, Katsanevakis S, Lipej L, Mastrototaro F, Mineur F, Pancucci-Papadopoulou MA, Ramos Espla A, Salas C, San Martin G, Sfrizo A, Streftaris N, Verlaque M (2012) A contribution to the application of European Union's marine strategy framework directive (MSFD). Part 2. Introduction trends and pathways. Mediterr Mar Sci 13(2):328–352.

Zhang LH, Dong YH (2004) Quorum sensing and signal interference: diverse implications. Mol Microbiol 53:1563–1571.

Zobell CE (1943) The effect of solid surfaces upon bacterial activity. J Bacteriol 43:39–56.

Žuljević A, Antolić B, Despalatovic M, Onofri V (2004) The spread of the invasive variety of *Caulerpa racemosa* in the Adriatic Sea. Rapports de la Commission Internationale pour l'Exploration Scientifique de la Mer Mediterranee 37:466.

2. Heterotrophic bacteria associated to the surface of *Caulerpa cylindracea* in the Mediterranean Sea

2.1. Abstract

Seaweed surfaces can harbour several microbial communities in marine ecosystems and, in turn, epiphytes could be a source of bioactive compounds able to protect the alga by their antibacterial activity. In the present study, epibacterial community on thalli of *Caulerpa cylindracea* (Sonder) was collected in the Marine Protected Area of Torre Guaceto (Brindisi, Italy) on the rocky shallow substrates of the Adriatic Sea during one sampling year. *Caulerpa cylindracea* is a non-indigenous species in the Mediterranean Sea, known as a great invasive macroalga able to colonize several types of substrates. Traditional cultivation with PCR-SSCP has been carried out and cultured and uncultured bacterial community have been compared to describe the microbial community associated to *C. cylindracea*. Molecular traces of gamma-Proteobacteria belonging to the genera *Shewanella* and *Vibrio* have been found by both approaches on the surface of *C. cylindracea* consistently in time, along with those of an unknown species belonging to the Rhodobacteraceae family. Other taxa belong to *Bacillus*, *Pseudoalteromonas*, *Tropicibacter*, *Photobacterium*, *Exiguobacterium*, *Kocuria*, *Ruegeria* and *Marinobacter* genera have been discovered with the culturable approach. On the other hand, PCR-SSCP method reveals traces of an unknown species of the Bacteroidetes phylum and the *Granulosicoccus* genus. Our results induce to hypothesize that *Caulerpa cylindracea* hosts a distinct microbial assemblage, with some taxa having a potential protective role for the alga. Further studies will clarify the nature of this algal-seaweed association and the possible role in the spreading of this alga using an holistic view considering the seaweed with associated bacteria as an essentially unique meta-organism.

2.2. Introduction

The seaweed surfaces offer a suitable substrate for the colonization of microorganisms. Several organic compounds utilized as nutrients for bacterial multiplication and microbial biofilm formation are also produced (Steinberg et al., 2002; Staufenberger et al., 2008; Singh, 2013). Algae-associated bacteria usually exchange signals with their hosts and are able to colonize algal surfaces and tissues to metabolize algal-derived carbon sources and to synthesize secondary metabolites (Preston et al., 1998). Although several researchers have investigated bacterial-algal interactions (e.g. Singh and Reddy, 2014), the ecological role of bacterial-algal associations is still unclear and the bacterial species often have not been identified (Margulis, 1981; Ashen et al., 1996). Apparently, the interactions between the algal host and the associated microbial community are critical not only for the growth and survival of the microbes themselves but may have important implications for the algal survival and for a suite of ecosystem processes (Azam et al., 1995; Paerl and Pinckney, 1996). Associated bacterial communities can produce bioactive compounds promoting algal growth, quorum sensing signals and other substances influencing algal morphology, development and survival (Singh and Reddy, 2014). Effects of epiphytic bacteria have been observed in several species of green algae such as *Ulv*a sp. (Provasoli, 1958), *Enteromorpha compressa* and *E. linz*a (Fries and Iwasaki, 1976) and *Monostroma oxyspermum* (Tatewaki et al., 1993).

Caulerpa has long been known to harbour microbial epiphytes, which may be related with various metabolic functions including nitrogen fixation and the production of several compounds (Meusnier et al., 2001). Traditionally, the first step to identify these organisms is their isolation and cultivation (Anonymous, 2013). However, it is well known that many microorganisms, especially bacteria, resist cultivation (Margulis et al., 1986; Pace, 1996). Furthermore, in several cases, algal surfaces harbour complex and diverse bacterial communities (Fisher et al., 1998). Microbial biofilms on the seaweed surfaces are indeed highly complex, dynamic and consist of a consortium including bacteria, fungi, diatoms, protozoa, spores and larvae of marine invertebrates (Lachnit et al., 2009; Goecke et al., 2010; Lachnit et al., 2011; Burke et al., 2011a, b). In this framework, the knowledge of the whole natural bacterial community on seaweed surfaces is critically needed. The molecular approaches based on PCR and the phylogenetics of the 16S rRNA gene are particularly useful (Pace, 1996). The sequence analysis of 16S ribosomal DNA (rDNA) allows the identification of unknown organisms to be utilized in

future investigations and might increase the information about the diversity of these interactions in the marine environment.

In the present study, we analyze the abundance and diversity of the heterotrophic bacterial community associated to the surface of the Mediterranean invasive alga *Caulerpa cylindracea* collected in a Mediterranean area (Brindisi, Adriatic Sea) and their variation in time. We complemented culture-based with molecular methods, which, as already recently reported by Guerrero-Ferreira et al. (2013), is a profitable approach to describe bacterial diversity. *Caulerpa cylindracea* (Sonder) (Belton et al., 2014), previously known as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman and Boudouresque (Verlaque et al., 2003), is a non-indigenous invasive algal species introduced in the Mediterranean Sea probably from the Red Sea and observed for the first time in Libya in 1990 (Klein and Verlaque, 2008). From then on, it has colonized all available habitats and substrata leading to profound structural and functional alterations of indigenous benthic assemblages and fish metabolism (Holmer et al., 2009; Cebrian et al., 2012; Felline et al., 2012). Aries et al. (2013) have already investigated, through tag-pyrosequencing, epiphytic and endophytic bacteria associated to *C. cylindracea* samples from the Mediterranean Sea and Australia, identifying the most prevalent orders, while, up to now, no data are available on the bacterial genera and species associated to this species. Our study was designed to assess: (1) if the diversity of bacterial community varies during the different algal developmental stages, and consequently (2) whether there is a specific unculturable and culturable microbial community associated to the seaweed. We discuss the ecological implications and the potential role of the microbial community in the spreading and habitat colonization of *C. cylindracea*.

2.3. Material and Methods

Sample collection

Caulerpa cylindracea was collected by SCUBA divers in the Marine Protected Area of Torre Guaceto (Brindisi, Italy) on rocky shallow substrates (5-10 m depth) (Fig. 2.1).

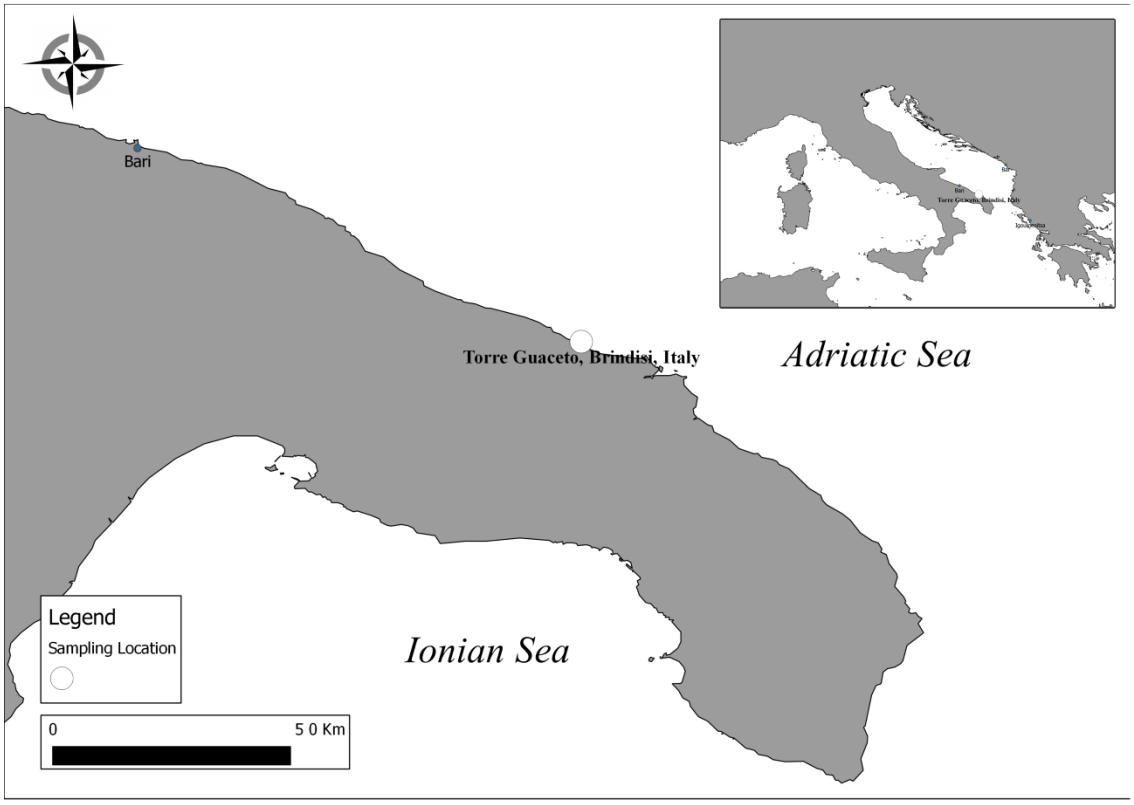


Figure 2.1. Map of the Apulian Region (Italy, Mediterranean Sea) showing the sampling point of *C. cylindracea*.

Caulerpa cylindracea shows a seasonal cycle with a period of vegetative growth approximately between June and November alternated with a period of vegetative rest (a quasi-complete withdrawal) approximately from December to May (Komatsu et al., 1997; Piazzi et al., 2001; Ruitton et al., 2005). During this vegetative rest period, despite the drop of cover and biomass, some stolons remain in the substrate, allowing a fast recolonization at the beginning of the following spring-summer. Samplings were carried out in four different times: September 2012 (time 1), May (time 2), July (time 3) and September 2013 (time 4). At each time, about 300 g of *C. cylindracea* were collected in triplicate and transferred to the laboratory under controlled temperature and processed for heterotrophic bacteria isolation and enumeration within 4 h from collection.

Bacteria enumeration

In the laboratory, the seaweed was washed several times in sterile seawater (0.2 μm pore filtered) to eliminate the bacteria settled on the surfaces, then suspended in sterile seawater and sonicated three times (Branson Sonifier 2200, 60 W, 47 kHz for 1 min in an ice bath) to optimize surface bacteria detachment. The sonication was interrupted for 30 s every minute, during which time the samples were shaken manually. To enumerate surface bacteria 1 or 5 mL of the sonicated sample and appropriate decimal dilutions were plated onto Marine Agar 2216 and after incubation for 7 days at 22 °C the culturable bacteria were counted according to the colony forming units (CFU) method.

Total bacterial counts were performed using a Zeiss Standard Axioplan microscope equipped with a halogen lA (Hg 100) light. Duplicate slides were prepared from each sample by filtering 1ml of seawater onto a Millipore filter (0.2 mm pore), using DAPI (4,6-diamidino-2 phenyl-indole) as fluorochrome (Porter and Feig, 1980). AG 365 excitation filter, an FT 395 chromatic beam splitter and an LP 420 barrier filter were used. At least 40 microscopic fields were counted for each preparation at 1000 magnification.

Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP), DNA sequencing and phylogenetic trees

Bacteria were collected from *C. cylindracea* (about 1 g) surface as described above, and high molecular weight genomic DNA was extracted as described (Vigliotta et al., 2005). After the extraction, the DNA was amplified using the bacteria-specific primers Com1-F (5'-CAGCAGCCGCGGTAATAC-3') and Com2-R (5'-CCGTCAATTCCTTTGAGTTT-3') targeting 16S rRNA encoding genes (Lane et al., 1985). These primers were designed to amplify 409 bp long DNA fragments (from nucleotide 519 to nucleotide 927 in the *Escherichia coli* 16S rRNA gene) that could be resolved by SSCP as described (Di Giacomo et al., 2007; Vigliotta et al., 2007; Stabili et al., 2008). To this purpose, PCR products were purified by High Pure PCR product Purification Kit (Boehringer Mannheim), denatured and resolved on 10% polyacrylamide gel (acrylamide/N,N-methylenebisacrylamide 49:1) in 0.8 X TBE (72 mM Tris-borate, 1.6 mM EDTA) containing 5% glycerol. Bands identified after silver staining were excised with razorblades and single strand DNAs were eluted from the gel by using the Qiaex II DNA purification kit (Qiagen). DNA similarity searches were carried out using

Ez-Taxon-e database (http:// (http://eztaxon-e.ezbiocloud.net, Kim et al., 2012). Sequence alignments were performed with ClustalW at EBI (http://www.ebi.ac.uk/). Phylogenetic analyses were conducted using the SeaView 4 program (Galtier et al., 1996; Gouy et al., 2010), according to the neighbour-joining (NJ) (Saitou and Nei, 1987), maximum-parsimony (MP) (Sober, 1983), and maximum-likelihood (ML) (Felsenstein, 1981) methods and Kimura's two-parameter algorithm (Kimura, 1980). Bootstrap analysis (Salemi and Vandamme, 2003) was used to estimate the reliability of phylogenetic reconstructions (1,000 replicates).

The 16S rDNA nucleotide sequences were deposited at GenBank with the following accession numbers: Bacteroidetes (KJ660319), *Shewanella* sp. (KJ660320), *Vibrio* sp. (KJ660322), *Granulosicoccus* sp. (KJ660323), Rhodobacteraceae (KJ660324).

16S rRNA Gene Sequence Analysis of Bacterial Isolates

High molecular weight genomic DNA from the different bacterial isolates was prepared according to standard procedures. Strains were grown in 100 mL nutrient broth (Difco) containing 3% NaCl with shaking at 30 °C to an optical density of 0.8 at 550 nm. After centrifugation, pellets were washed with 50 mL of STE buffer [500 mM NaCl, 50 mM Tris–Cl (pH 8), 5 mM EDTA], and then resuspended in 4 mL of a solution containing 50 mM Tris–Cl (pH 8), 25% sucrose, 1 mM EDTA. Lysozyme (1 mg mL^{-1}) treatment was carried out at 0 °C for 10 min, then EDTA was added to a final concentration of 40 mM, and samples were incubated at 0 °C for 10 min. Proteinase K (100 mg mL^{-1}) treatment was performed for 2 h at 65 °C after addition of sodium dodecyl sulfate (SDS) to a final concentration of 1 %. Nucleic acids were extracted by phenol–chloroform/isoamylic alcohol (24:1) extraction according to standard procedures (Sambrook and Russell, 2001), and 15 µg mL^{-1} ribonuclease A were used to remove RNA. After phenol–chloroform/isoamylic alcohol (24:1) extraction and ethanol precipitation, high molecular weight DNA was collected by spooling using Shepherd's crooks (Sambrook and Russell, 2001).

The 16S rRNA-encoding genes were amplified using the Eubacterial-specific primers 16SEB20-43-F (5′-TGGCTCAGATTGAACGCTGGCGG-3′) and 16SEB683-R (5′-CTACGCATTTCACCGCTACAC -3′) (Vigliotta et al., 2007; Talà et al., 2013). The two primers were designed to amplify a 683 bp-long DNA fragment (from nucleotide 20 to

nucleotide 702 in the *Escherichia coli* 16S rRNA gene). PCR reactions were performed as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 55 °C and extension at 72 °C for 1-2 min, and the final elongation step at 72 °C for 5 min. They were carried out in a Perkin-Elmer Cetus DNAThermal Cycler 2400. PCR products were isolated through 1%agarose gels in 1X TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0), recovered using the Qiaex II Gel extraction kit (Qiagen) and finally sequenced as a service by MWG Biotech Custom Sequencing Service (Germany). The sequences of all isolates were compared with those of closely related reference strains using the EzTaxon-e server (Kim et al., 2012). Multiple sequence alignments were performed with CLUSTAL W (Thompson et al., 1994) at the Kyoto University Bioinformatic Center (http://www.genome.jp/tools/clustalw/) using the following default settings. The CLUSTAL W output file was used to construct evolutionary trees with the SeaView software (Galtier et al., 1996; Gouy et al., 2010) in accordance with the neighbour-joining (Saitou and Nei, 1987), maximum-parsimony (Sober, 1983) and maximum-likelihood (Felsenstein, 1981) methods. Evolutionary distances were calculated with the neighbor-joining method in accordance with the algorithm of Kimura's two parameter model (Kimura, 1980). Tree robustness was assessed by bootstrap resampling (1,000 replicates each) (Brown, 1994). The nucleotide sequence of almost the entire 16S rRNA-encoding gene of isolates was deposited at GenBank with the following accession numbers: KM189196 (CR-T1-H1), KM189197 (CR-T1-H2), KM189198 (CR-T1-H3), KM189199 (CR-T1-H4), KM189200 (CR-T1-H5), KM189201 (CR-T1-H6), KM189202 (CR-T1-H7), KM189203 (CR-T4-H33), KM189204 (CR-T1-H8), KM189205 (CR-T1-H9), KM189206 (CR-T2-H10), KM189207 (CR-T2-H11), KM189208 (CR-T2-H12), KM189209 (CR-T2-H13), KM189210 (CR-T2-H14), KM189211 (CR-T2-H15), KM189212 (CR-T2-H16), KM189213 (CR-T3-H17), KM189214 (CR-T3-H18), KM189215 (CR-T3-H19), KM189216 (CR-T3-H20), KM189217 (CR-T3-H21), KM189218 (CR-T3-H22), KM189219 (CR-T3-H23), KM189220 (CR-T3-H24), KM189221 (CR-T4-H25), KM189222 (CR-T4-H26), KM189223 (CR-T4-H27), KM189224 (CR-T4-H28), KM189225 (CR-T4-H29), KM189226 (CR-T4-H30), KM189227 (CR-T4-H31), KM189228 (CR-T4-H32).

2.4. Results

Microbiological Analyses and Isolation of bacteria in Pure Cultures

Total bacteria and culturable bacteria densities on the surface of *C. cylindracea* in the four sampling times are reported in Fig. 2.2.a. In particular, in time 1, on average, culturable heterotrophic bacteria accounted for 3.2 x 10^6 CFU mL^{-1}, in time 2 their density reached 5.1 x 10^5 CFU mL^{-1}, 2.0 x 10^6 CFU mL^{-1} in time 3 and 2.9 x 10^6 CFU mL^{-1} in time 4. The trends of bacterial abundance during sampling periods are shown in Fig. 2.2.b. The lowest bacterial concentration is reported in May, the highest one is found in September.

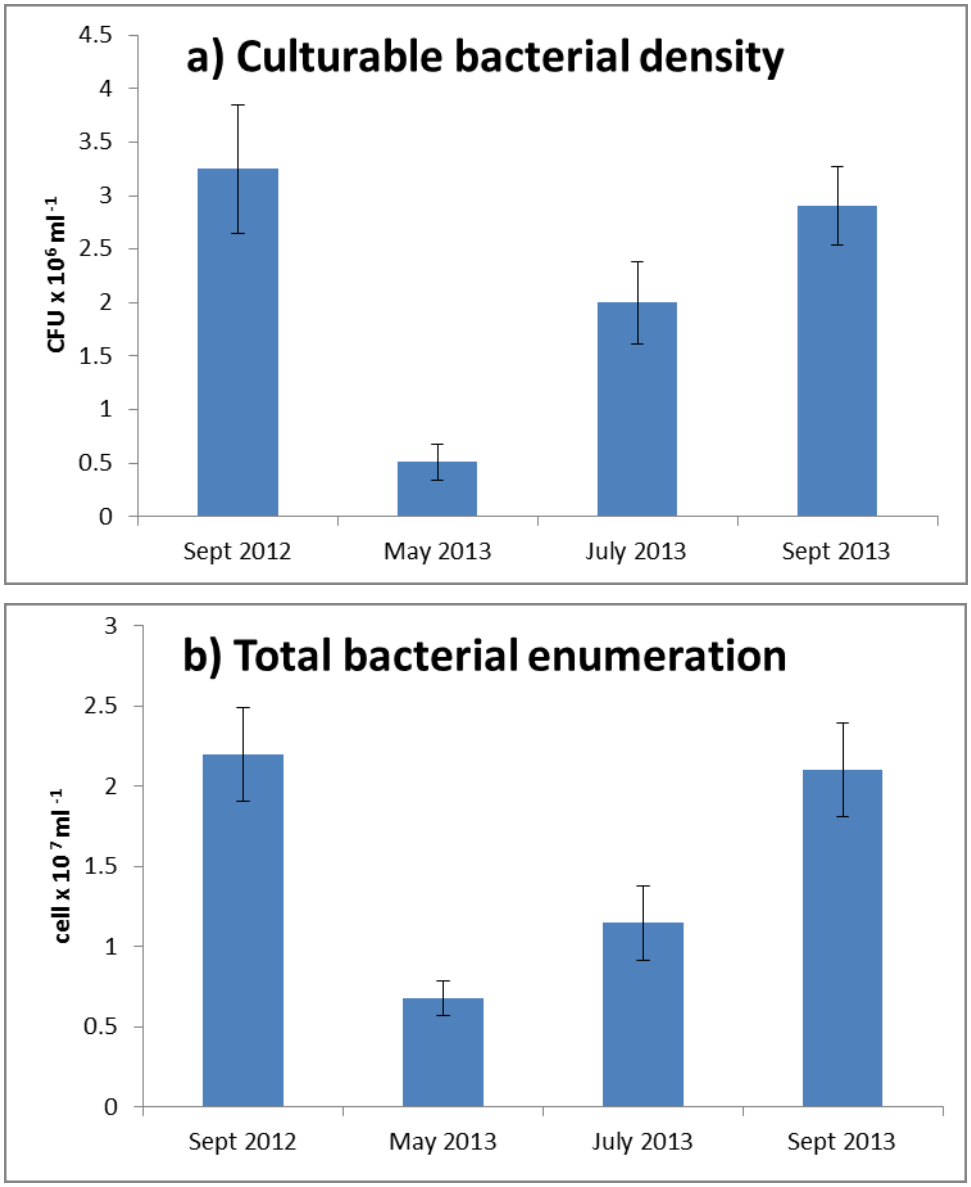


Figure 2.2. Mean abundance and relative standard deviation of a) cultural heterotrophic bacterial densities and b) total bacteria numeration on *C. cylindracea* collected in the four sampling times.

PCR-SSCP, DNA sequencing and phylogenetic trees

The epiphytic bacterial communities of *C. cylindracea* sampled in four sampling times were analyzed by PCR–SSCP using bacteria-specific primers Com1-F and Com2-R. These primers target a 409-bp-long (in *Escherichia coli*) central region of prokaryotic 16S rRNA gene and allow an high taxonomic resolution. The SSCP profile of the amplified 16S rRNA gene pool revealed a relatively low degree of complexity of the microbial communities living on *C. cylindracea* surface with a predominance of a few common bands among samples examined (Fig. 2.3). In particular bands **a**, **b**, **c**, **d** and **e** were common to all the samples.

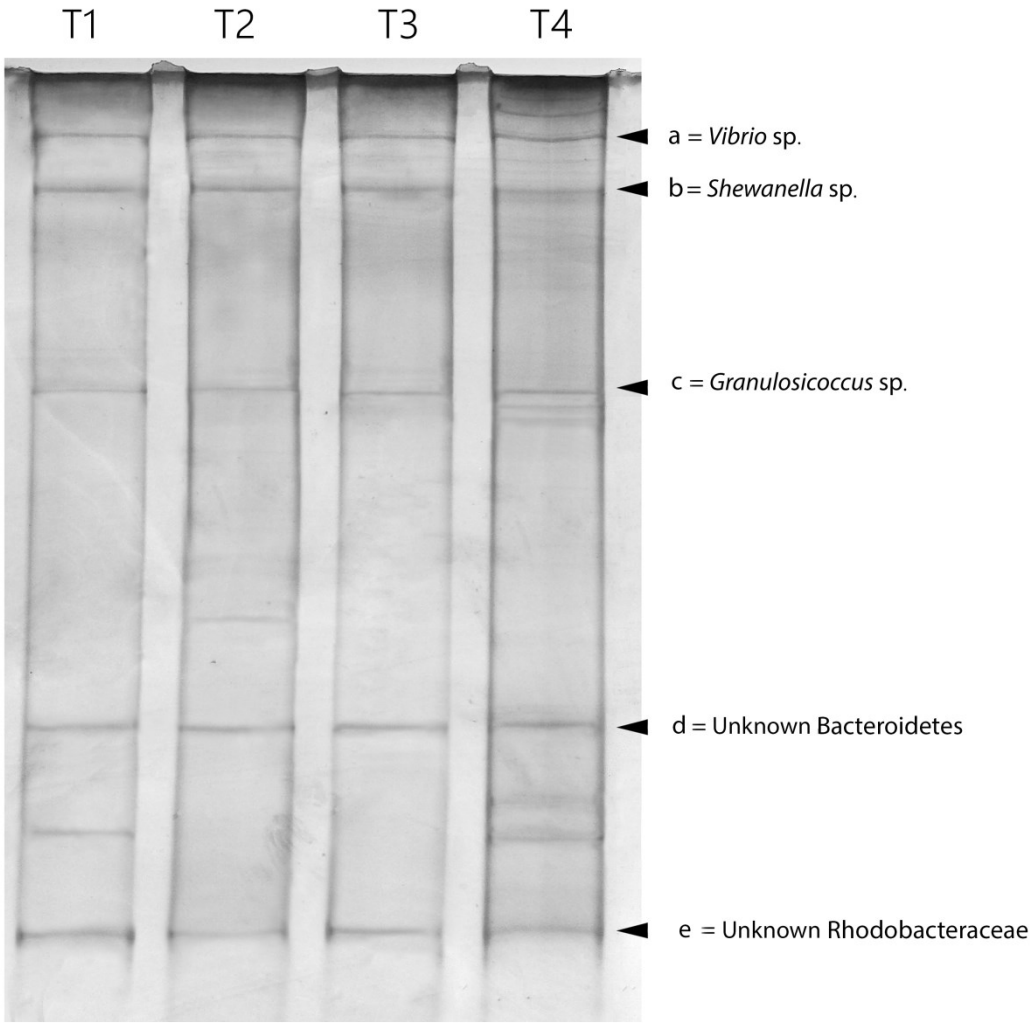


Figure 2.3. PCR–SSCP analysis of the bacterial community on *C. cylindracea* surface. Four distinct batches of *C. cylindracea* sampled in Marine Protected Area of Torre Guaceto were analyzed for the presence of epibiotic bacteria by PCR–SSCP using 16S rRNA gene-specific primers. Arrowheads mark the positions of specific DNA bands (a, b, c, d, e).

All the above mentioned bands were excised from the gel, eluted, and subjected to DNA sequencing. The Ez-Taxon analysis of the DNA sequences demonstrated that the bacteria common to all four *C. cylindracea* samples belonged to the genera *Granulococcoides, Shewanella* and *Vibrio. Granolosicoccus coccoides* (91.57 % identity), *Vibrio sagamiensis* and *Vibrio rotiferianus* (99.71% identity) both belonging to the *V. harveyi* clade were the most closely-related species as also shown by phylogenetic tree analysis (Fig. 2.4).

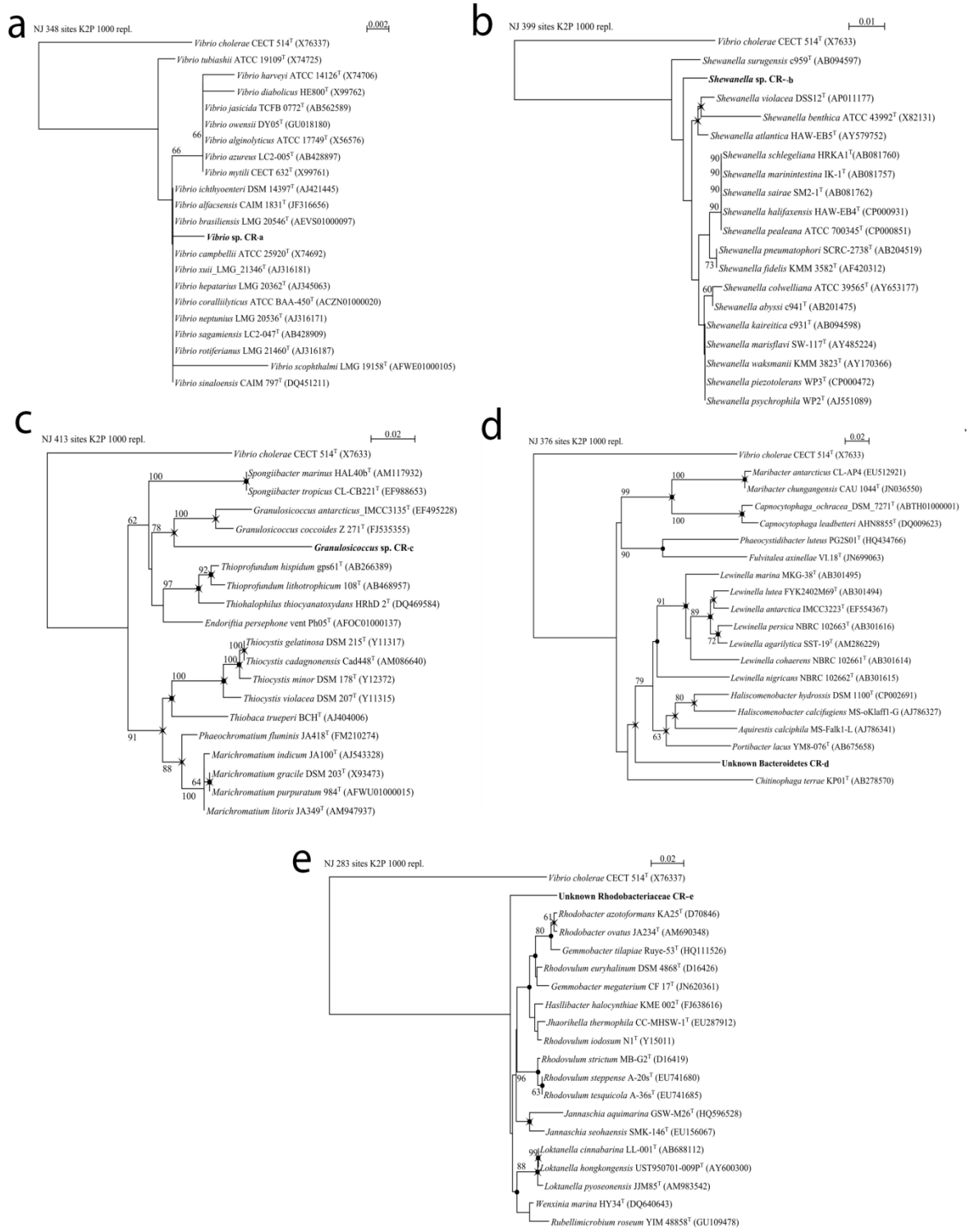


Figure 2.4. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing positions of (a) *Vibrio,* (b) *Shewanella*, (c) *Granulosicoccus* genera; Bacteroidetes phylum (d) and Rhodobacteraceae family (e) from *C. cylindracea* surface (in bold) with respect to closely-related species. Bootstrap values (expressed as percentages of 1,000 replicates) of >60 % are shown at branch point. Filled circles and "X" indicate that corresponding nodes were also recovered in phylogenetic trees constructed with maximum parsimony and maximum-likelihood algorithms respectively.

As regards *Shewanella* the most closely-related species belonged to a robust cluster that enclosed *Shewanella waksmanii*, *Shewanella marisflavi*, *Shewanella pealeana*, *Shewanella piezotolerans*, *Shewanella halifaxensis, Shewanella violacea*, *Shewanella kaireitica*, *Shewanella schlegeliana*, *Shewanella marinintestina*, *Shewanella sairae* and *Shewanella psychrophila* (98.75 % identity) (Fig. 2.4). Band **d**, was assigned to an unknown species of Bacteroidetes phylum (87.40 % identity with *Lewinella marina*) (Fig. 2.4), and band **e** was assigned to a microorganism belonging to Rhodobacteraceae family (96.11% identity with *Wenxinia marina*) (Fig. 2.4).

Identification of heterotrophic bacteria isolates from *C. cylindracea* surface

For each sampling time, a total of sixty heterotrophic bacteria isolates were preliminarily analysed and grouped, based on their cultural and biochemical properties, into nine types for the first sampling time, seven types for second sampling time, eight types for the third sampling time and nine types for the fourth time. Representative isolates from each of the type groups were characterized by nucleotide sequencing of 16S-rRNA-encoding genes. These isolates were designated with the abbreviation CR, indicating the source of the isolation, followed by two numbers one preceded by a letter T referred to the sampling time and one preceded by a letter H (heterotrophic bacteria) referred to the sequential number.

In Table 2.1 the cuturable isolates in each of the four sampling times are reported. The overall heterotrophic bacteria isolated from *C. cylindracea* surface were: *Bacillus litoralis*, *Pseudoalteromonas peptidolytica*, *Shewanella waksmanii*, *Vibrio* spp., *Tropicibacter multivorans*, *Photobacterium gaetbulicola*, *Exiguobacterium marinum*, *Kocuria rosea*, *Ruegeria arenilitoris* and *Marinobacter xestospongiae*. *Bacillus litoralis*, *Pseudoalteromonas peptidolytica*, *Shewanella waksmanii*, *Vibrio* spp. were recorded in all the sampling times.

Table 2.1. Heterotrophic diversity on *C. cylindracea* surface collected in the four sampling times

	I	II	III	IV
Vibrio neptunius	+	+	+	+
Vibrio sp.	+	+	+	+
Vibrio jasicida	+	+	+	+
Shewanella waksmanii	+	+	+	+
Bacillus litoralis	+	+	+	+
Pseudoarteromonas peptidolytica	+	+	+	+
Tropicibacter multivorans	+	-	+	+
Photobacterium gaetbulicola	-	+	-	+
Marinobacter xestospongiae	-	-	-	+
Exiguobacterium marinum	+	-	-	-
Kocuria rosea	+	-	-	-
Rugeria arenolitis	-	-	+	-

In particular EzTaxon-e analysis of the 16S rRNA gene sequence of *Bacillus* CR-T1-H5, CR-T2-H15, CR-T3-H22 and CR-T4-H29 indicated that the closest relative of these strains (100% identity) was *Bacillus litoralis* SW-211^T (Yoon and Oh, 2005). In the neighbour-joining tree, CR-T1-H5, CR-T2-H15, CR-T3-H22 and CR-T4-H29 branched together with the aforesaid reference strain (Fig. 2.5). The relationship between CR-T1-H5, CR-T2-H15, CR-T3-H22 and CR-T4-H29 and *Bacillus litoralis* SW-211^T (Yoon and Oh, 2005) was also maintained in trees constructed with the maximum-parsimony and maximum-likelihood algorithms.

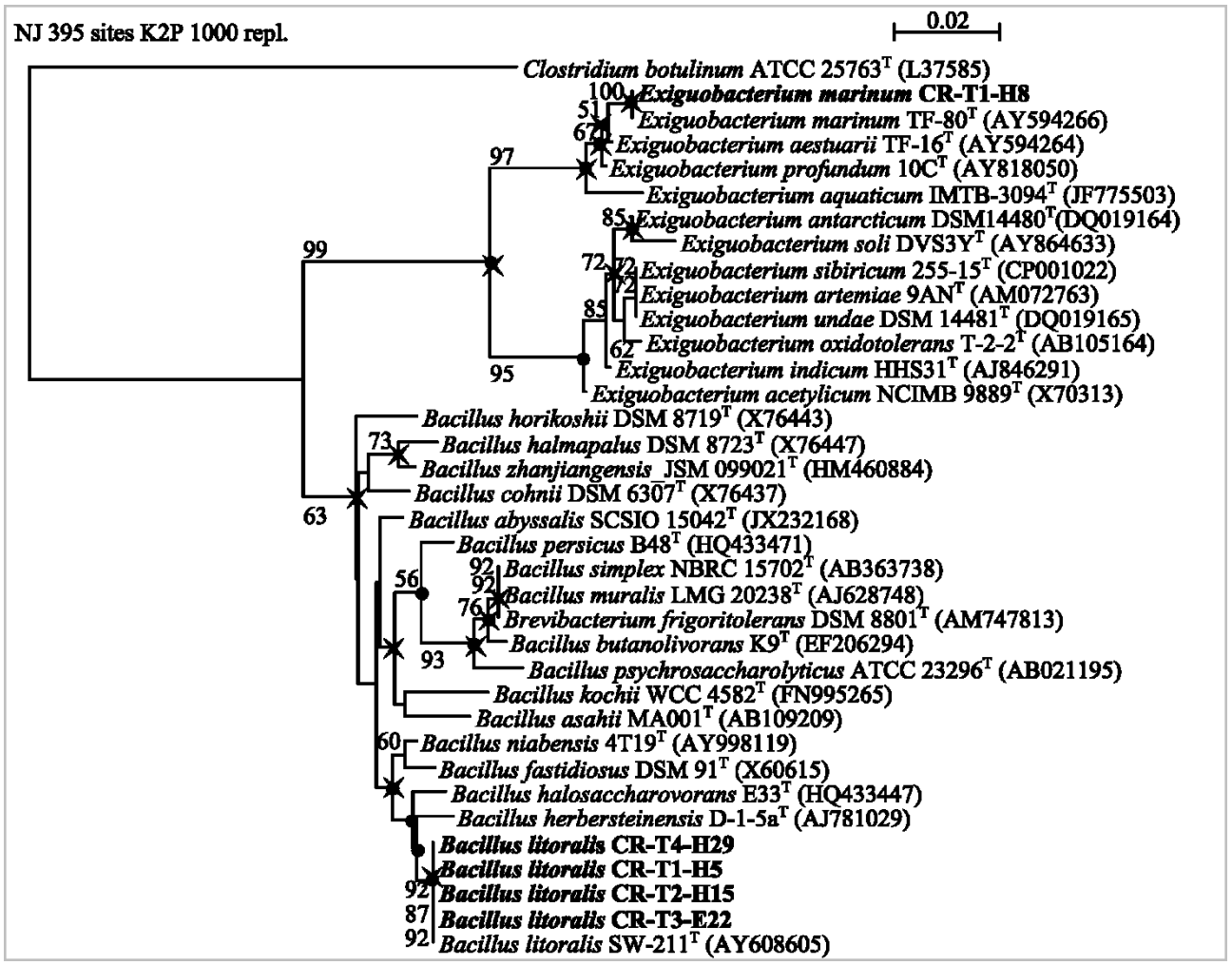


Figure 2.5. Neighbour-joining phylogenetic trees based on 16S rRNA gene sequences showing positions of *Exiguobacterium marinum* and *Bacillus litoralis* (*Clostridium botulinum* ATCC 257^T was used as outgroup) isolated from *C. cylindracea* surface (in bold) with respect to closely-related species. Bootstrap values (expressed as percentages of 1,000 replicates) of >50 % are shown at branch point. Filled circles and "X" indicate that corresponding nodes were also recovered in phylogenetic trees constructed with maximum parsimony and maximum-likelihood algorithms respectively.

The 16S rRNA gene sequence of *Pseudoalteromonas peptidolytica* CR-T1-H6, CR-T2-H16, CR-T3-H23 and CR-T4-H30 (100% sequence identity) appeared to be closely related to that of the reference strain *Pseudoalteromonas peptidolytica* F-12-50-A1^T (Venkateswaran and Dohmoto, 2000) a novel marine mussel-thread-degrading bacterium isolated from the Sea of Japan. These isolates clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees (Fig. 2.6) together with *Pseudoalteromonas peptidolytica* F-12-50-A1^T (Venkateswaran and Dohmoto, 2000).

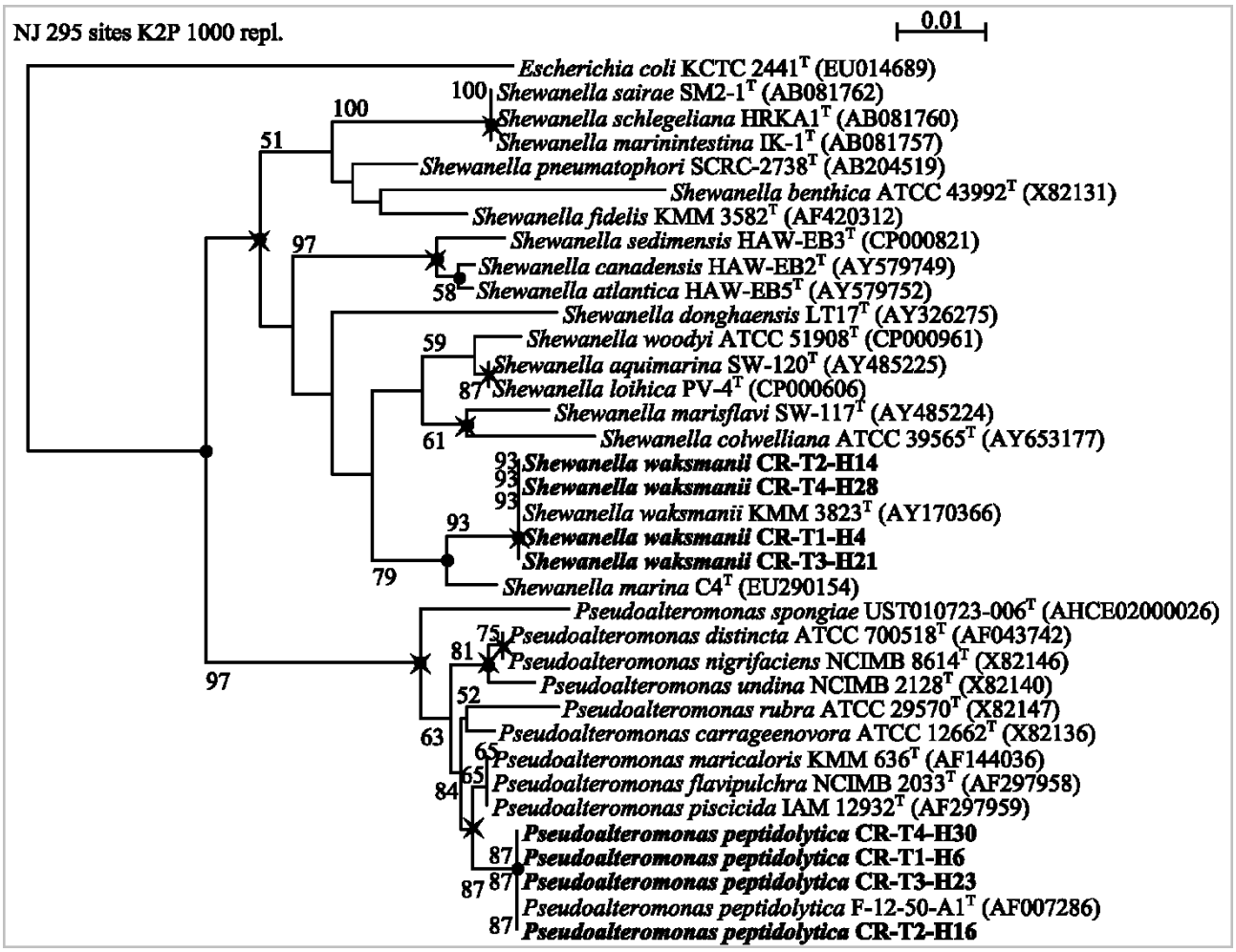


Figure 2.6. Neighbour-joining phylogenetic trees based on 16S rRNA gene sequences showing positions of *Shewanella waksmanii* and *Pseudoalteromonas peptidolytica* (*Escerichia coli* KCTC 2441^T was used as outgroup) isolated from *C. cylindracea* surface (in bold) with respect to closely-related species. Bootstrap values (expressed as percentages of 1,000 replicates) of >50 % are shown at branch point. Filled circles and "X" indicate that corresponding nodes were also recovered in phylogenetic trees constructed with maximum parsimony and maximum-likelihood algorithms respectively.

The 16S rRNA gene sequence of isolates CR-T1-H4, CR-T2-H14, CR-T3-H21 and CR-T4-H28 seemed to be close to that of the reference strain *Shewanella waksmanii* KMM 3823^T (Ivanova et al., 2003), isolated from a sipuncula *Phascolosoma japonicum* (100% identity). These isolates clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees (Fig. 2.6) together with *Shewanella waksmanii* KMM 3823^T (Ivanova et al., 2003).

EzTaxon-e analysis of the 16S rRNA gene sequence of *Vibrio* sp. CR-T1-H1, CR-T2-H11, CR-T3-H18 and CR-T4-H25 revealed an high identity (100%) with the 16S rRNA

gene sequence of *V. neptunius* type strain LMG 20536^T (Thompson et al., 2003). In the neighbour-joining, maximum-parsimony and maximum-likelihood trees (Fig. 2.7) they grouped together with *V. neptunius* LMG 20536^T (Thompson et al., 2003).

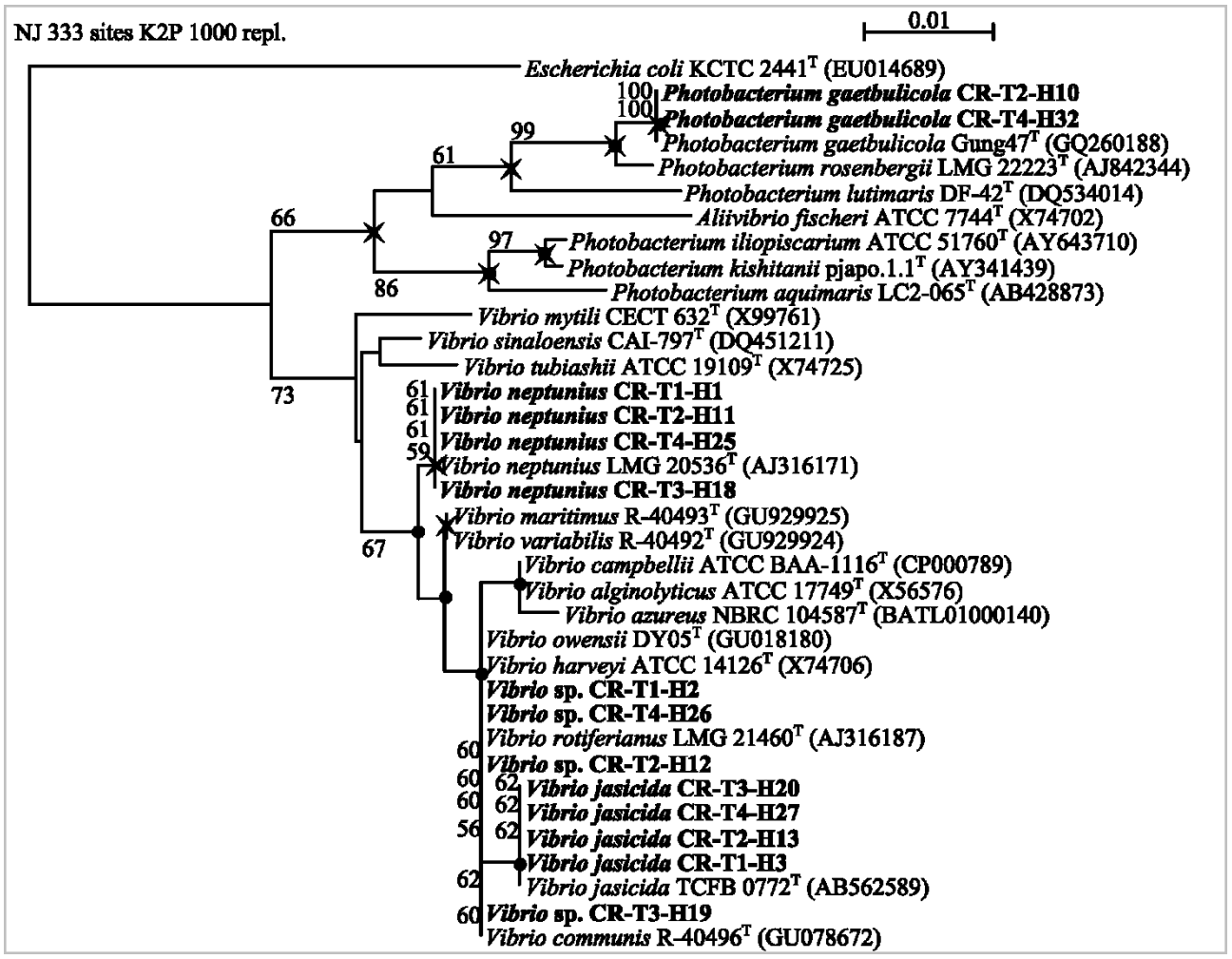


Figure 2.7. Neighbour-joining phylogenetic trees based on 16S rRNA gene sequences showing positions of *Photobacterium gaetbulicola*, *Vibrio neptunius*, *Vibrio jasicida* and *Vibrio* sp. (*Escerichia coli* KCTC 2441^T was used as outgroup) from *C. cylindracea* surface (in bold) with respect to closely-related species. Bootstrap values (expressed as percentages of 1,000 replicates) of >50 % are shown at branch point. Filled circles and "X" indicate that corresponding nodes were also recovered in phylogenetic trees constructed with maximum parsimony and maximum-likelihood algorithms respectively.

The 16S rRNA gene sequences of *Vibrio* sp. CR-T1-H3, CR-T2-H13, CR-T3-H20 and CR-T4-H27 isolates showed an identity of 100% with the homologous gene sequence of *V. jasicida* TCFB 0772^T (Yoshizawa et al., 2012), isolated from several marine animals. In the neighbour-joining, maximum-parsimony and maximum-likelihood trees (Fig. 2.7)

the four isolates clustered together with the reference strain *V. jasicida* TCFB 0772^T (Yoshizawa et al., 2012).

EzTaxon-e analysis of the 16S rRNA gene sequence of *Vibrio* sp. CR-T1-H2 and CR-T2-H12 demonstrated an identity of 100% with the homologous sequence of *V. rotiferianus* LMG 21460^T (Gomez-Gil et al., 2003), while CR-T3-H19 and CR-T4-H26 demonstrated an high identity with *V. rotiferianus* LMG 21460^T (Gomez-Gil et al., 2003), *V. owensii* DY05^T (Cano-Gomez et al., 2010) and *Vibrio communis* R-40496^T (Chimetto et al., 2011). In the neighbour-joining and maximum-parsimony phylogenetic trees (Fig. 2.7), CR-T1-H2, CR-T2-H12, CR-T3-H19 and CR-T4-H26 isolates branched with *V. rotiferianus* LMG 21460^T (Gomez-Gil et al., 2003), *V. owensii* DY05^T (Cano-Gomez et al., 2010), *Vibrio communis* R-40496^T (Chimetto et al., 2011) and *Vibrio harveyi* ATCC 14126^T (Johnson and Shunk, 1936; Hendrie et al., 1970; Baumann et al., 1980).

Tropicibacter multivorans was recognized in sampling times 1, 3 and 4. The isolates CR-T1-H7 CR-T3-H24 and CR-T4-H31 showed an high identity with *Tropicibacter multivorans* MD5^T (Lucena et al., 2012). These isolates clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees (Fig. 2.8) together with *Tropicibacter multivorans* MD5^T (Lucena et al., 2012).

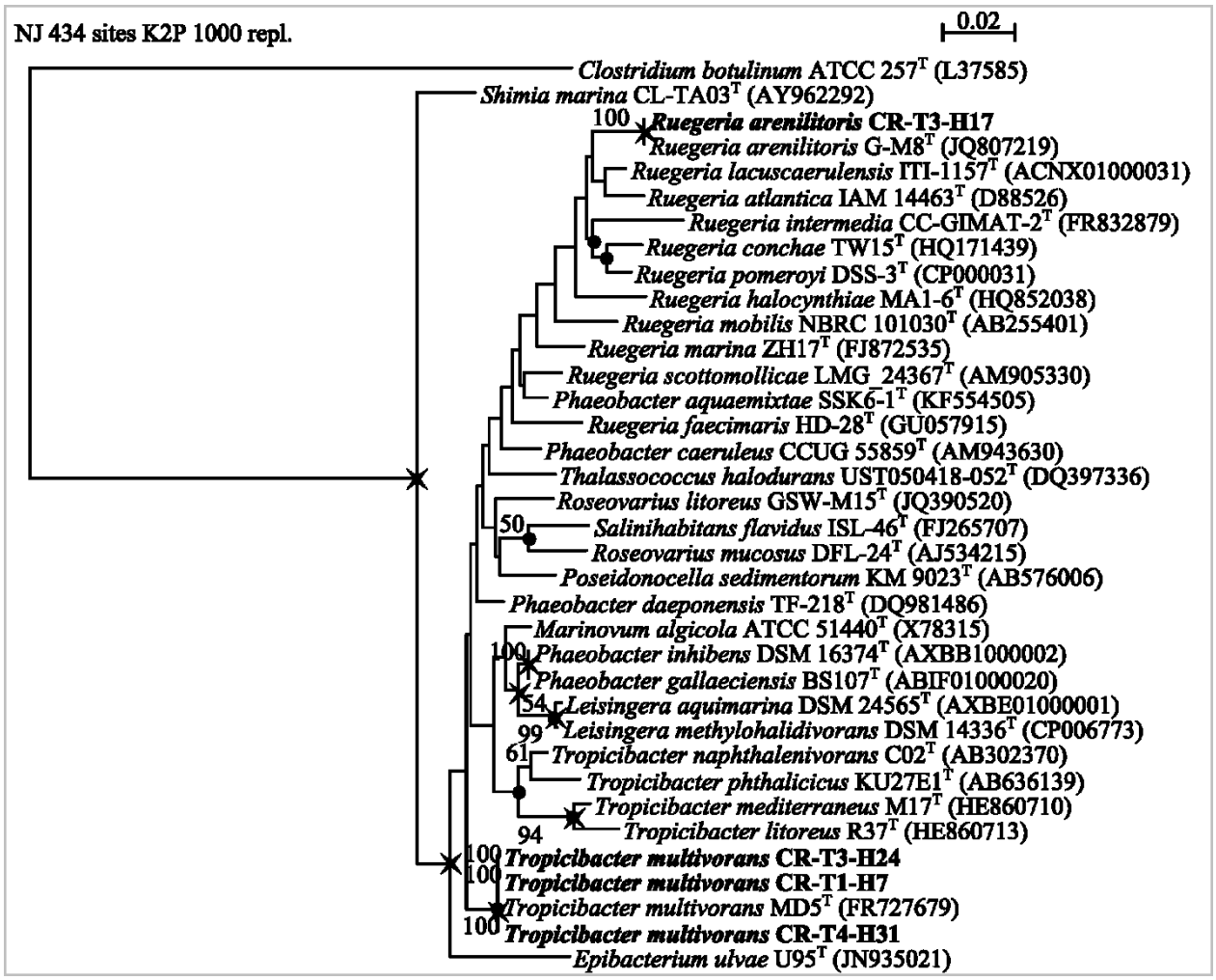


Figure 2.8. Neighbour-joining phylogenetic trees based on 16S rRNA gene sequences showing positions of *Ruegeria arenilitoris* and *Tropicibacter multivorans* (*Clostridium botulinum* ATCC 257^T was used as outgroup) isolated from *C. cylindracea* surface (in bold) with respect to closely-related species. Bootstrap values (expressed as percentages of 1,000 replicates) of >50 % are shown at branch point. Filled circles and "X" indicate that corresponding nodes were also recovered in phylogenetic trees constructed with maximum parsimony and maximum-likelihood algorithms respectively.

Photobacterium sp. CR-T2-H10, CR-T4-H33 recorded in sampling times 2 and 4 showed an identity of 100% with the *Photobacterium gaetbulicola* type strain Gung47^T (Kim et al., 2010). These isolates clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees (Fig. 2.7) together with *Photobacterium gaetbulicola* Gung47^T (Kim et al., 2010).

Some bacterial species were isolated only one time during the study period. In particular, the sampling time 1 the 16S rRNA gene sequences of isolates CR-T1-H8 showed an identity of 100% with the homologous gene sequence of *Exiguobacterium marinum* TF-

80^T (Kim et al., 2005), isolated from a tidal flat of the Yellow Sea in Korea. This isolate clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees (Fig. 2.5) together with *Exiguobacterium marinum* TF-80^T (Kim et al., 2005).

Kocuria rosea was identified only in sampling times 1, demonstrating an identity of 100% with the homologous sequence of *Kocuria rosea* type strain DSM 20447^T (Flügge, 1886; Brooks and Murray, 1981; Poston, 1993; Stackebrandt et al., 1995; Schumann et al., 1999; Rainey et al., 1997; Schumann et al., 2000). This isolate clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees (Fig. 2.9) together with *Kocuria rosea* type strain DSM 20447^T (Flügge, 1886; Brooks and Murray, 1981; Poston, 1993; Stackebrandt et al., 1995; Rainey et al., 1997; Schumann et al., 1999; Schumann et al., 2000).

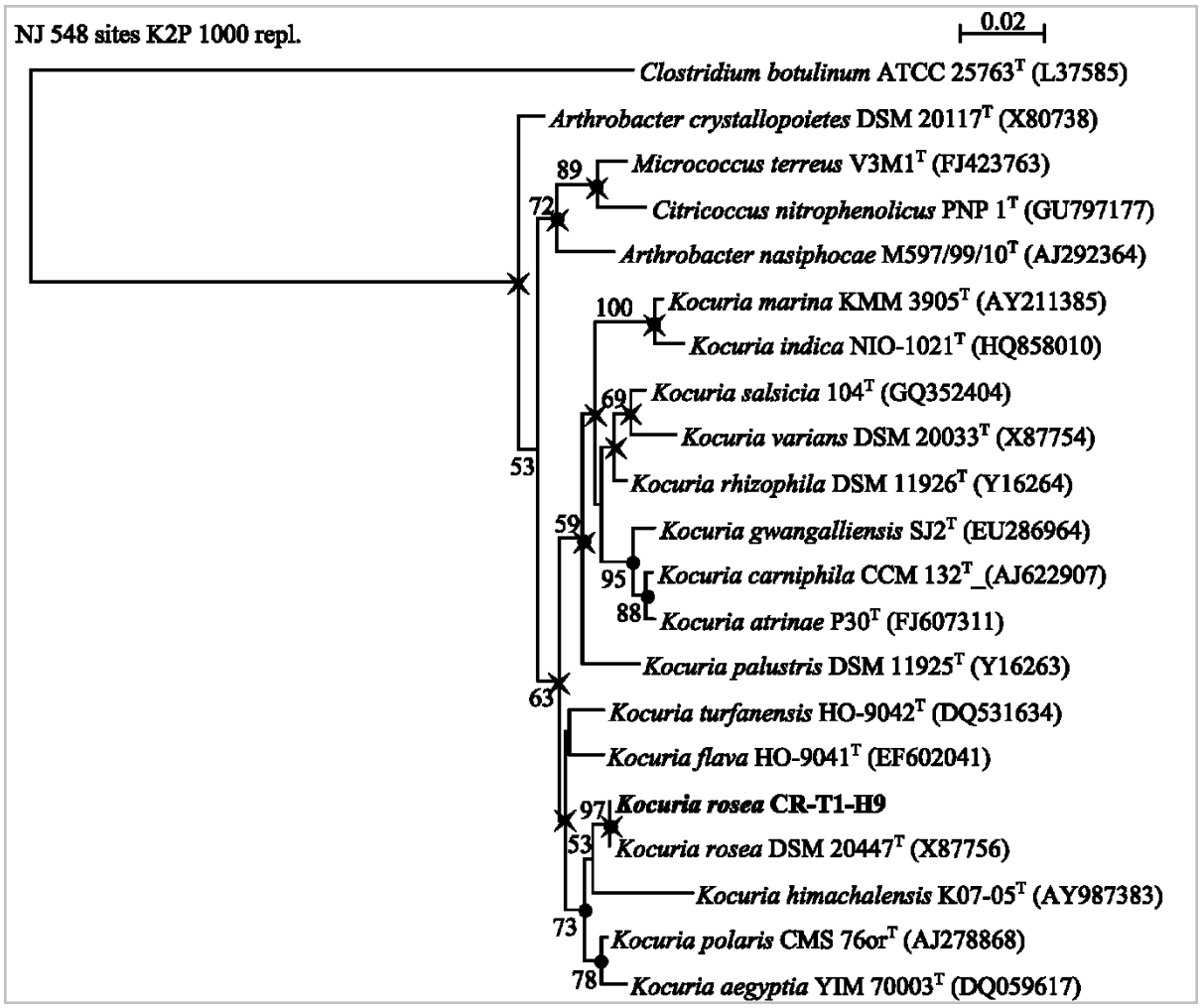


Figure 2.9. Neighbour-joining phylogenetic trees based on 16S rRNA gene sequences showing positions of *kocuria rosea* (*Clostridium botulinum* ATCC 257^T was used as outgroup) isolated from *C. cylindracea* surface (in bold) with respect to closely-related species. Bootstrap values (expressed as percentages of 1,000 replicates) of >50 % are shown at branch point. Filled circles and "X" indicate that corresponding nodes were also recovered in phylogenetic trees constructed with maximum parsimony and maximum-likelihood algorithms respectively.

In the third sampling time the 16S rRNA gene sequence of isolate CR-T3-H17 appeared to be closely related to that of the reference strain *Ruegeria arenilitoris* G-M8^T (Park and Yoon, 2012), the neighbour-joining, maximum-parsimony and maximum-likelihood trees confirmed this result (Fig. 2.8).

Finally, the 16S rRNA gene sequence of isolate CR-T4-H33 showed an identity of 100%to the reference strain *Marinobacter xestospongiae* UST090418-1611^T (Lee et al., 2012). This isolate clustered in the neighbour-joining, maximum-parsimony and

maximum-likelihood trees (Fig. 2.10) together with *Marinobacter xestospongiae* UST090418-1611^T (Lee et al., 2012).

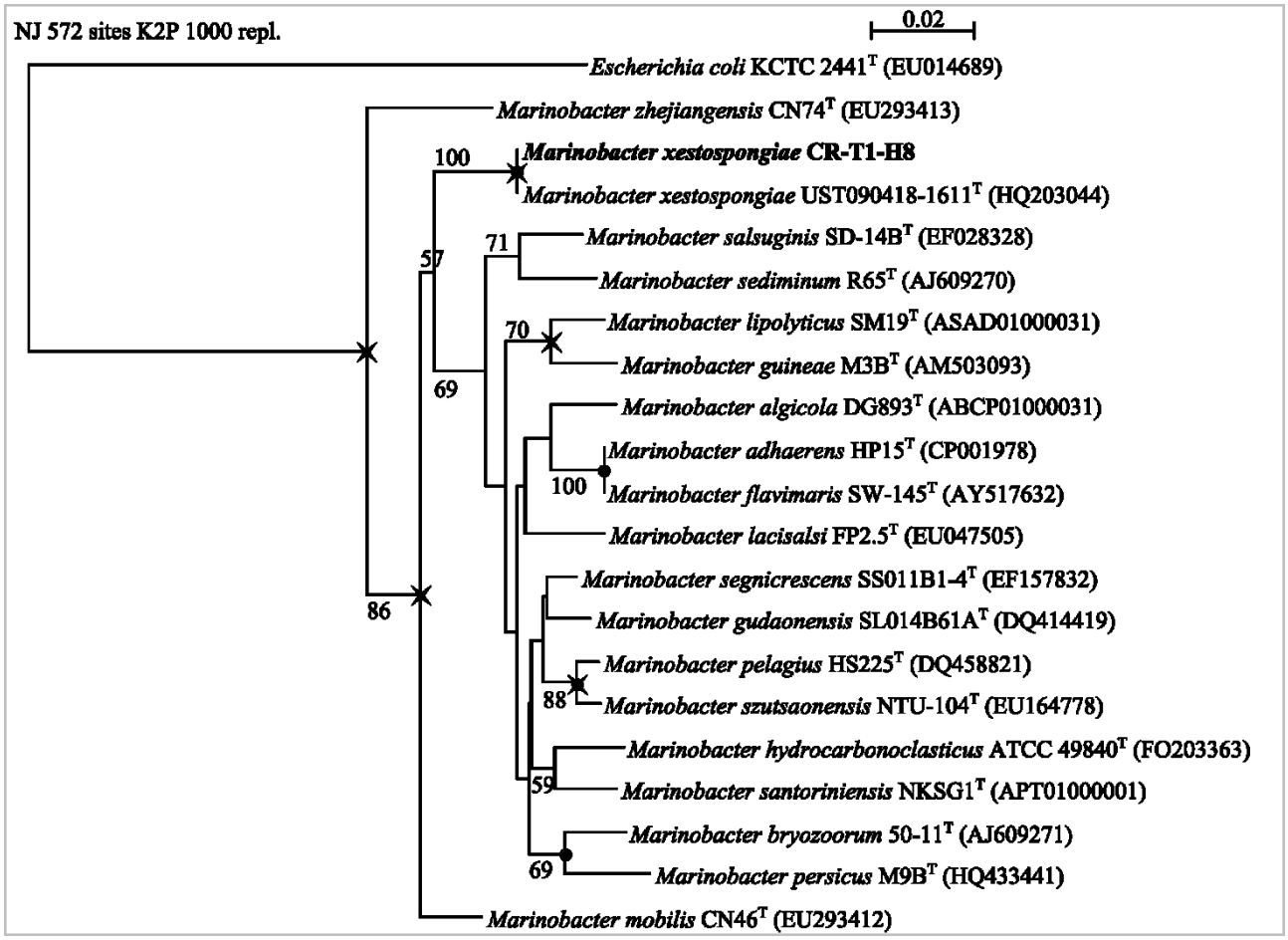


Figure 2.10. Neighbour-joining phylogenetic trees based on 16S rRNA gene sequences showing positions of *Marinobacter xestospongiae* (*Escerichia coli* KCTC 2441^T was used as outgroup) isolated from *C. cylindracea* surface (in bold) with respect to closely-related species. Bootstrap values (expressed as percentages of 1,000 replicates) of >50 % are shown at branch point. Filled circles and "X" indicate that corresponding nodes were also recovered in phylogenetic trees constructed with maximum parsimony and maximum-likelihood algorithms respectively.

2.5. Discussion

The present work represent the first attempt to characterize, at genus/species level, the bacterial community associated to the invasive alga *C. cylindracea* collected in a coastal area of Mediterranean Eastern basin.

On the surface of *C. cylindracea*, we recorded the presence of gamma-Proteobacteria belonging to the genera *Shewanella* and *Vibrio*, along with an unknown species belonging to the Rhodobacteraceae family consistently in time, by complementing culture-based with unculturable PCR-SSCP methods. These results are in accordance with Aries et al. (2013) detecting, through tag-Pyrosequencing, gamma-Proteobacteria and alfa-Proteobacteria, in particular Rhodobacterales, among the prevalent taxa of epiphytic bacteria associated on *C. cylindracea*. Moreover, we found, through PCR-SSCP, traces of an unknown species of the Bacteroidetes phylum and the *Granulosicoccus* genus in all the investigation periods. Although in most of cases the lengths of the amplified 16S rRNA sequences and their homologies in the databank were not sufficient to assign the putative species name to each PCR–SSCP bands, the sequence of band **c** exhibited considerable identity (99.51%) with that of *Granulosiccoccus coccoides* Z 271^T (Kurilenko et al., 2010). This species is isolated from leaves of seagrass, *Zostera marina* in Pacific Ocean. Related sequences of *Granulosicoccus* genus (Lee et al., 2007) have been also found on the red seaweed *Delisea pulchra*, the green seaweed *Ulva australis* (Longford et al., 2007), the brown alga *Fucus vesiculosus* (Lachnit et al., 2011) and on the kelp *Laminaria saccharina* (Staufenberger et al., 2008), suggesting these bacteria share a predilection for seaweeds (Bengtsson et al., 2012).

Both utilized approaches highlighted some bacterial strains belonging to the Harveyi clade, which includes some potential pathogens to benthic organisms (Urbanczyk et al., 2013). In particular, and the analysis of these 16S r-RNA sequences of the strains CR-T1-H2, CR-T2-H12, CR-T3-H19 and CR-T4-H26 isolated from *C. cylindracea* surface showed a great identity percentage with *V. rotiferianus* LMG 21460^T (Gomez-Gil et al., 2003), *V. owensii* DY05^T (Cano-Gomez et al., 2010) and *Vibrio communis* R-40496^T (Chimetto et al., 2011) and the neighbour-joining and maximum-parsimony phylogenetic trees confirmed these results. In particular, *V. rotiferianus* was isolated from cultures of the rotifer *Brachionus plicatilis* (Gomez-Gil et al., 2003) and is known as a marine pathogen of various aquatic organisms (Chen et al., 2011). Interestingly this bacterial strain associated with the brown macroalga *Delesseria sanguinea* exhibited an

antibacterial activity against some of the bacteria exclusively associated with the macroalga (Goecke et al., 2013a,b). *Vibrio communis* was isolated from the marine corals *Mussismilia hispida* and *Phyllogorgia dilatata*, the zoanthids *Palythoa caribaeorum* and *Palythoa variabilis* and the Pacific white shrimp *Litopenaeus vannamei* (Chimetto et al., 2011), while *V. owensii* is an invertebrate pathogen isolated for the first time from diseased cultured crustaceans *Panulirus ornatus* and *Penaeus monodon* in Australia and has been recognized as a pathogen of marine-reared crustaceans (Cano-Gómez et al., 2010; Goulden et al., 2012). Isolates CR-T1-H3, CR-T2-H13, CR-T3-H20 and CR-T4-H27, found from *C. cylindracea* surface throughout the year, seem to be closely related to *Vibrio jasicida*, another member of the Harveyi clade, isolated from packhorse lobster, abalone and Atlantic salmon (Yoshizawa et al., 2012). In the phylogenetic trees, CR-T1-H1, CR-T2-H11, CR-T3-H18 and CR-T4-H25 clustered together with *V. neptunius,* a species already isolated from larvae of the bivalve *Nodipecten nodosus* in the South of Brazil (Thompson et al., 2003) and documented as a molluscan pathogen (Prado et al., 2005).

Noteworthy, both methods evidenced the presence of strains belonging to *Shewanella* genus in all sampling times. The genus *Shewanella* (MacDonell and Colwell, 1985) was frequently isolated from aquatic habitats and also from clinical sources (MacDonell and Colwell, 1985; Gauthier et al., 1995; Venkateswaran et al., 1999). The members of this genus represent one of the most numerically abundant microbes among cultivated marine proteobacteria. Some species have been widely investigated due to their importance in co-metabolic bioremediation of halogenated organic pollutants (Petrovskis et al., 1994), crude petroleum (Semple and Westlake, 1987), the manganese and iron oxides reduction (Myers and Nealson, 1988) and polyunsaturated fatty acids production (Russel and Nichols, 1999). In particular, the isolates CR-T1-H4, CR-T2-H14, CR-T3-H21 and CR-T4-H28 seemed to be related to the reference strain *Shewanella waksmanii* KMM 3823^T (Ivanova et al., 2003), found in the sipuncula *Phascolosoma japonicum* from the Pacific Ocean.

It is well known that the culture-based approach is able to capture more members of the rare biosphere than omic methods (Shade et al., 2012). In the present study, by using culturable approach, we have identified other isolates not detected through PCR-SSCP such as *Bacillus*, *Pseudoalteromonas Tropicibacter*, *Photobacterium*, *Exiguobacterium*, *Kocuria*, *Ruegeria* and *Marinobacter* genera. The *Bacillus* genus has a great

heterogeneity in physiology, ecology, and genetics (Slepecky and Hemphill, 2006). The range of physiological life styles is impressive: degraders of most all substrates derived from plant and animal sources, antibiotic producers, heterotrophic nitrifiers, denitrifiers, nitrogen fixers, iron precipitators, selenium oxidizers, oxidizers and reducers of manganese, facultative chemolithotrophs, acidophiles, alkalophiles, psychrophiles, thermophiles and others (Slepecky, 1972; Norris et al., 1981; Claus and Berkeley, 1986). Strains CR-T1-H5, CR-T2-H15, CR-T3-H22 and CR-T4-H29 indicated that their closest relative type strain was *Bacillus litoralis* SW-211^T (Yoon and Oh, 2005) already isolated from a tidal flat of the Yellow Sea in Korea.

Pseudoalteromonas genus includes several species which are successful settlers of living surfaces, often associated with other organisms (Dheilly et al., 2010), with a potential protective role of their host from fouling thanks to the production of bioactive compounds (Egan et al., 2000). Several species of *Pseudoalteromonas*, indeed, produce bioactive compounds with antibacterial, antifungal, or antiviral activity (Holmström et al., 2002) that tackle a broad range of marine biofilms (Dheilly et al., 2010) and fouling organisms (Holmström et al., 1998; Holmström et al., 2002). A recent study evaluates the antifouling potential of *P. piscicida*, *P. tunicata*, and *P. ulvae* in a system mimicking the natural marine environment and under real-life conditions (Bernbom et al., 2013), concluding that *Pseudoalteromonas* spp. early operate as bacterial attractants in mesocosms of coastal waters but have subsequent antifouling ability. The strains CR-T1-H6, CR-T2-H16, CR-T3-H23 and CR-T4-H30 appeared to be closely related to type strain *Pseudoalteromonas peptidolytica* F-12-50-A1^T (Venkateswaran and Dohmoto, 2000) isolated from the surface waters of the Yamato Islands, in the Sea of Japan. The extracellular products of this species are responsible for the degradation of the non-degradable peptide present in the permanent adhesive in the foot of marine invertebrate *Mytilus edulis*, thus presumably playing an important role as antifouler.

Culturable dependent analysis revealed also the presence of *Tropicibacter* genus in three out of the four sampling times. *Tropicibacter* is a bacterial genus under Rhodobacteraceae family, already found in all samples by PCR-SSCP method. In particular the type strain closest to the isolates CR-T1-H7 CR-T3-H24 and CR-T4-H31 is *Tropicibacter multivorans* MD5^T (Lucena et al., 2012), an aerobic marine alpha-proteobacterium, isolated from Mediterranean seawater in Spain. The strain was placed phylogenetically within the *Roseobacter* clade, an abundant group of bacteria known in marine

bacterioplankton worldwide and acting a central role in pelagic sulfur cycling. A recent study revealed that *Roseobacter* clade bacteria are an integral part of the microbial community in marine sediments, where they may oxidize inorganic and organic sulfur compounds in oxic sediments (Lenk et al., 2012). However, up to now, little information is available on their abundance and function in benthic ecosystem.

Members of the genus *Photobacterium* have been isolated from marine organisms, marine sediments, seawater and saline lakes (Seo et al., 2005; Thompson et al., 2005; Farmer and Hickman-Brenner, 2006; Park et al., 2006; Rivas et al., 2006; Ast et al., 2007). *Photobacterium* sp. CR-T2-H10, CR-T4-H33, recorded in two of four sampling times, were related to *Photobacterium gaetbulicola* type strain Gung47^T (Kim et al., 2010), a lipolytic bacterium isolated from a tidal flat sediment.

Finally, only few of the isolated isolates (*Marinobacter* sp., *Exiguobacterium* sp., *kocuria* sp., *Ruegeria* sp.) have a sporadic presence on the algal surface whilst most of the isolates were found in all the four sampling times. On account of these results we may exclude that bacterial epibionts found on *C. cylindracea* surface tend to be associated with highly similar epibiotic microbial communities (Staufenberger et al., 2008; Lachnit et al., 2009). Our results induce to hypothesize that *C. cylindracea* hosts a distinct microbial assemblage. Recently, Wahl et al. (2012), stated the need to use a more holistic view, considering the seaweed with associated bacteria as an essentially unique meta-organism, from a physical, functional and evolutional point of view. In this framework, the role of the bacterial isolates on the surface of *C. cylindracea* needs to be clarified to understand their potential effect on the patterns of colonization.

2.6. References

Aires T, Serrão EA, Kendrick G, Duarte CM, Arnaud-Haond S (2013) Invasion Is a Community Affair: Clandestine Followers in the Bacterial Community Associated to Green Algae, *Caulerpa racemosa*, Track the Invasion Source. PLoS ONE 8(7):e68429. doi:10.1371/journal.pone.0068429

Anonymous (2013) The cultural revolution. Nat Rev Microbiol 11:1.

Ashen JB, Goff LJ (1996) Molecular identification of a bacterium associated with gall formation in the marine red alga Priontis lanceolata. J Phycol 32:286–297.

Ast JC, Cleenwerck I, Engelbeen K, Urbanczyk H, Thompson FL, De Vos P, Dunlap PV (2007) *Photobacterium kishitanii* sp. nov., a luminous marine bacterium symbiotic with deep-sea fishes. Int J Syst Evol Microbiol 57:2073–2078.

Azam F, Smith DC, Long RA, Steward GF (1995) Bacteria in oceanic carbon cycling as a molecular problem, p. 39–54. In I. Joint (ed.), Molecular ecology of aquatic microbes. Springer-Verlag, Berlin, Germany.

Baumann P, Baumann L, Bang SS, Woolkalis MJ (1980). Reevaluation of the taxonomy of *Vibrio*, *Beneckea*, and *Photobacterium*: abolition of the genus *Beneckea*. Curr Microbiol 4(3):127-132.

Belton GS, Prud'homme van Reine, WF, Huisman JM, Draisma SGA, Gurgel CFD (2014) Resolving phenotypic plasticity and species designation in the morphology challenging *Caulerpa racemosa-peltata* complex (Caulerpaceae, Chlorophyta). J Phycol 50(1):32-54.

Bengtsson MM, Sjøtun K, Lanzén A, Øvreås L (2012) Bacterial diversity in relation to secondary production and succession on surfaces of the kelp *Laminaria hyperborea*. ISME J 6(12):2188-2198.

Bernbom N, Ng YY, Olsen SM, Gram L (2013) Pseudoalteromonas spp. Serve as Initial Bacterial Attractants in Mesocosms of Coastal Waters but Have Subsequent Antifouling Capacity in Mesocosms and when Embedded in Paint. Appl Environ Microbiol 79(22):6885-6893.

Brooks BW, Murray RGE (1981) Nomenclature for "*Micrococcus radiodurans*" and other radiation-resistant cocci: Deinococcaceae fam. nov. and *Deinococcus* gen. nov., including five species. Int J Syst Bacteriol 31(3):353-360.

Brown JK (1994) Bootstrap hypothesis tests for evolutionary trees and other dendrograms. Proc Natl Acad Sci USA 91:12293–12297.

Burke C, Thomas T, Lewis M, Steinberg P, Kjelleberg S (2011a) Composition, uniqueness and variability of the epiphytic bacterial community of the green alga *Ulva australis*. ISME J 5:590–600.

Burke C, Steinberg P, Rusch D, Kjelleberg S, Thomas T (2011b) Bacterial community assembly based on functional genes rather than species. P Natl Acad Sci USA 108:14288–14293.

Cano-Gómez A, Goulden EF, Owens L, Høj L (2010) *Vibrio owensii* sp. nov., isolated from cultured crustaceans in Australia. FEMS Microbiol Lett 302(2):175-81.

Cebrian E, Linares C, Marchal C, Garrabou J (2012) Exploring the effects of invasive algae on the persistence of gorgonian populations. Biol Invasions 14:2647–2656.

Chen Z, Yao Z, Lin M, Chang J (2011) Isolation and identification of *Vibrio rotiferianus* from diseased half-smooth tongue sole (*Cynoglossus semilaevis* Günther). In Biomedical Engineering and Informatics (BMEI), 2011 4th International Conference on (Vol. 4, pp. 2267-2272). IEEE.

Chimetto LA, Cleenwerck I, Alves N, Silva BS, Brocchi M, Willems A, De Vos P, Thompson FL (2011) *Vibrio communis* sp. nov., isolated from the marine animals *Mussismilia hispida*, *Phyllogorgia dilatata*, *Palythoa caribaeorum*, *Palythoa variabilis* and *Litopenaeus vannamei*. Int J Syst Evol Microbiol 61(2):362-368.

Claus DRC, Berkeley W (1986) The genus *Bacillus*. 1105– 1139. P. H. A. Sneath (ed.) Bergey's manual of systematic bacteriology, vol. 2. Williams and Wilkins. Baltimore.

Dheilly A, Soum-Soutéra E, Klein GL, Bazire A, Compére C, Haras D, Dufour A (2010) Antibiofilm activity of the marine bacterium *Pseudoalteromonas* sp. strain 3J6. Appl Environ Microbiol 76:3452–3461.

Di Giacomo M, Paolino M, Silvestro D, Vigliotta G, Imperi F, Visca P, Alifano P, Parente D (2007) Microbial community structure and dynamics of dark fire-cured tobacco fermentation. Appl Environ Microbiol 73:825–837.

Egan S, Thomas T, Holmstrom C, Kjelleberg S (2000) Phylogenetic relationship and antifouling activity of bacterial epiphytes from the marine alga *Ulva lactuca*. Environ Microbiol 2:343–347.

Farmer J, III Hickman-Brenner F (2006) The genera *Vibrio* and *Photobacterium*. In The Prokaryotes, 3rd edn, vol. 6, pp. 508–563. Edited by M Dworkin, S Falkow, E Rosenberg, KH Schleifer, E Stackebrandt. New York: Springer.

Felline S, Caricato R, Cutignano A, Gorbi S, Lionetto MG, Mollo E, Regoli F, Terlizzi A (2012) Subtle effects of biological invasions: cellular and physiological responses of fish eating the exotic pest *Caulerpa racemosa*. PLoS ONE 7:e38763. doi:10.1371/journal.pone.0038763.

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17: 368–376.

Fisher MM, Wilcox LW, Graham LE (1998) Molecular characterization of epiphytic bacterial communities on charophycean green algae. Appl Environ Microbiol. 64(11):4384-4389.

Flügge C (1886) Die Mikroorganismen: Mit besonderer Berücksichtigung der Aetiologie der Infectionskrankheiten. Leipzig: F.C.W. Vogel.

Fries L, Iwasaki H (1976) P-hydroxyphenylacetic acid and other phenolic compounds as growth stimulators of the red alga Porphyra tenera. Pl Sci Lett 6:299–307.

Gauthier G, Gauthier M, Christen R (1995) Phylogenetic analysis of the genera *Alteromonas*, *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. Int J Syst Bacteriol 45:755–761.

Galtier N, Gouy M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12:543–538.

Goecke F, Labes A, Wiese J, Imhoff JF (2010) Chemical interactions between marine macroalgae and bacteria. Mar Ecol-Prog Ser 409:267–300.

Goecke F, Labes A, Wiese J, Imhoff JF (2013a) Phylogenetic analysis and antibiotic activity of bacteria isolated from the surface of two co-occurring macroalgae from the Baltic Sea. Eur J Phycol 48(1):47-60.

Goecke F, Thiel V, Wiese J, Labes A, Imhoff JF (2013b) Algae as an important environment for bacteria – phylogenetic relationships among new bacterial species isolated from algae. Phycologia 52:14–24.

Gomez-Gil B, Thompson FL, Thompson CC, Swings J (2003) *Vibrio rotiferianus* sp. nov., isolated from cultures of the rotifer Brachionus plicatilis. Int J Syst Evol Microbiol 53(1):239-243.

Goulden EF, Hall MR, Bourne DG, Pereg LL, Høj L (2012) Pathogenicity and infection cycle of *Vibrio owensii* in larviculture of the ornate spiny lobster (*Panulirus ornatus*). Appl Environ Microbiol 78(8):2841-2849.

Gouy M, Guindon S, Gascuel O (2010) SeaView Version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27 (2):221-224.

Guerrero-Ferreira R, Gorman C, Chavez AA, Willie S, Nishiguchi MK (2013) Characterization of the bacterial diversity in Indo-West Pacific loliginid and sepiolid squid light organs. Microb Ecol 65(1):214-226.

Hendrie MS, Hodgkiss W, Shewan JM (1970) The Identification, Taxonomy and Classification of Luminous Bacteria. J Gen Microbiol 64(2):151-169.

Holmer M, Marbà N, Lamote M, Duarte CM (2009) Deterioration of Sediment Quality in Seagrass Meadows (*Posidonia oceanica*) Invaded by Macroalgae (*Caulerpa* sp.). Estuaries Coasts 32:456-466.

Holmström C, James S, Neilan BA, White DC, Kjelleberg S (1998) *Pseudoalteromonas tunicata* sp. nov., a bacterium that produces antifouling agents. Int J Syst Bacteriol 48:1205–1212.

Holmström C, Egan S, Franks A, McCloy S, Hjelleberg S (2002) Antifouling activities expressed by marine surface associated *Pseudoalteromonas* species. FEMS Microbiol Ecol 41:47–58.

Ivanova EP, Nedashkovskaya OI, Zhukova NV, Nicolau DV, Christen R, Mikhailov VV (2003) *Shewanella waksmanii* sp. nov., isolated from a sipuncula (*Phascolosoma japonicum*). Int J Syst Evol Microbiol 53(5):1471-1477.

Johnson FH, Shunk IV (1936) An interesting new species of luminous bacteria. J Bacteriol 31(6):585.

Kim IG, Lee MH, Jung SY, Song JJ, Oh TK, Yoon JH (2005) *Exiguobacterium aestuarii* sp. nov. and *Exiguobacterium marinum* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. Int J Syst Evol Microbiol 55(2):885-889.

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721.

Kim YO, Kim KK, Park S, Kang SJ, Lee JH, Lee SJ, Oh TK, Yoon JH (2010) *Photobacterium gaetbulicola* sp. nov., a lipolytic bacterium isolated from a tidal flat sediment. Int J Syst Evol Microbiol 60(11):2587-2591.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120.

Klein J, Verlaque M (2008) The *Caulerpa racemosa* invasion: a critical review. Mar Pollut Bull 56:205–225.

Komatsu T, Meinesz A, Buckles D (1997) Temperature and light responses of alga *Caulerpa taxifolia* introduced into the Mediterranean Sea. Mar Ecol-Prog Ser 146:145–153.

Kurilenko VV, Christen R, Zhukova NV, Kalinovskaya NI, Mikhailov VV, Crawford RJ, Ivanova EP (2010) *Granulosicoccus coccoides* sp. nov., isolated from leaves of seagrass (*Zostera marina*). Int J Syst Evol Microbiol 60(4):972-976.

Lachnit T, Blumel M, Imhoff JF, Wahl M (2009) Specific epibacterial communities on macroalgae: phylogeny matters more than habitat. Aquat Biol 5:181–186.

Lachnit T, Meske D, Wahl M, Harder T, Schmitz R (2011) Epibacterial community patterns on marine macroalgae are host-specific but temporally variable. Environ Microbiol 13:655–665.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA 82:6955–6959.

Lee OO, Lai PY, Wu HX, Zhou XJ, Miao L, Wang H, Qian PY (2012) *Marinobacter xestospongiae* sp. nov., isolated from the marine sponge *Xestospongia testudinaria* collected from the Red Sea. Int J Syst Evol Microbiol 62(8):1980-1985.

Lee K, Lee HK, Choi T-H, Kim K-MT, Cho J-C (2007) Granulosicoccaceae fam. nov., to include *Granulosicoccus antarcticus* gen. nov., sp. nov., a non-phototrophic, obligately aerobic chemoheterotroph in the order Chromatiales, isolated from antarctic Seawater. J Microbiol Biotechnol 17:1483–1490.

Lenk S, Moraru C, Hahnke S, Arnds J, Richter M, Kube M, Reinhardt R, Brinkhoff Harder TJ, Amann R, Mußmann M (2012) Roseobacter clade bacteria are abundant in coastal sediments and encode a novel combination of sulfur oxidation genes. ISME J 6(12):2178-2187.

Longford SR, Tujula NA, Crocetti GR, Holmes AJ, Holmström C, Kjelleberg S, Steinberg PD, Taylor MW (2007) Comparisons of diversity of bacterial communities associated with three sessile marine eukaryotes. Aquat Microb Ecol 48:217–229.

Lucena T, Pujalte MJ, Ruvira MA, Garay E, Macián MC, Arahal DR (2012) *Tropicibacter multivorans* sp. nov., an aerobic alphaproteobacterium isolated from surface seawater. Int J Syst Evol Microbiol 62(4):844-848.

MacDonell MT, Colwell RR (1985) Phylogeny of the Vibrionaceae, and recommendation for two new genera, *Listonella* and *Shewanella*. Syst Appl Microbiol 6:171–182.

Margulis L (1981) Symbiosis in cell evolution, p. 164. W. H. Freeman and Co., San Francisco, Calif.

Margulis L, Chase D, Guerrero R (1986) Microbial communities. Bioscience 36:160–170.

Meusnier I, Olsen JL, Stam WT, Destombe C, Valero M (2001) Phylogenetic analyses of *Caulerpa taxifolia* (Chlorophyta) and of its associated bacterial microflora provide clues to the origin of the Mediterranean introduction. Mol Ecol 10(4):931-946.

Myers CR, Nealson KH (1988) Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science 240:1319–1321.

Norris JRR, Berkeley W, Logan NA, O'Donnell AG (1981) The genera *Bacillus* and *Sporalactobacillus*. 1711–1742. MP Starr, A Stolp, AG Truper, A Balows, HG Schlegel (ed.) The prokaryotes, vol. 2. Springer-Verlag. Berlin.

Pace NR (1996) New perspective on the natural microbial world: molecular microbial ecology. ASM News 62:463–470.

Paerl HW, Pinckney JL (1996) A mini-review of microbial consortia: their roles in aquatic production and biogeochemical cycling. Microb Ecol 31:225–247.

Park S, Yoon JH (2012) *Ruegeria arenilitoris* sp. nov., isolated from the seashore sand around a seaweed farm. A Van Leeuw 102(4):581-589.

Park YD, Baik KS, Seong CN, Bae KS, Kim S, Chun J (2006) *Photobacterium ganghwense* sp. nov., a halophilic bacterium isolated from sea water. Int J Syst Evol Microbiol 56:745–749.

Petrovskis EA, Vogel TM, Adriaens P (1994) Effects of electron acceptors and donors on transformation of tetrachloromethane by *Shewanella putrefaciens* MR-1. FEMS Microbiol Lett 121:357–364.

Piazzi L, Ceccherelli G, Cinelli F (2001) Threat to macroalgal diversity: effects of the introduced green alga *Caulerpa racemosa* in the Mediterranean. Mar Ecol-Progr Ser 210:161–165.

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25:943–948.

Poston JM (1993). *Pelczaria aurantia* gen. nov., sp. nov., a newly described orange-colored bacterium. Arch Microbiol 160(2):114-120.

Prado S, Romalde JL, Montes J, Barja JL (2005) Pathogenic bacteria isolated from disease outbreaks in shellfish hatcheries. First description of *Vibrio neptunius* as an oyster pathogen. Dis Aquat Org 67: 209-215.

Preston GM, Haubold B, Rainey PR (1998) Bacterial genomics and adaptation to life on plants: implications for the evolution of pathogenicity and symbiosis. Curr Opin Microbiol 1:589-597.

Provasoli L (1958) Effect of plant hormones on *Ulva*. Biol Bull 114:375–384.

Rainey FA, Nobre MF, Schumann P, Stackebrandt E, da Costa MS (1997) Phylogenetic diversity of the deinococci as determined by 16S ribosomal DNA sequence comparison. Int J Syst Bacteriol 47(2):510-514.

Rivas R, Garcıa-Fraile P, Mateos PF, Martınez-Molina E, Velazquez E (2006) *Photobacterium halotolerans* sp. nov., isolated from Lake Martel in Spain. Int J Syst Evol Microbiol 56:1067–1071.

Ruitton S, Verlaque M, Boudouresque CF (2005) Seasonal changes of the introduced *Caulerpa racemosa* var. *cylindracea* (Caulerpales, Chlorophyta) at the northwest limit of its Mediterranean distribution. Aquat Bot 82: 55–70.

Russell NJ, Nichols DS (1999) Polyunsaturated fatty acids in marine bacteria – a dogma rewritten. Microbiology 145:767–779.

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Salemi M, Vandamme AM (2003) The phylogenetic handbook: a practical approach to DNA and protein phylogeny. Cambridge University Press, Cambridge, United Kingdom.

Sambrook J, Russell DW (2001) Molecular Cloning. A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Schumann P, Spröer C, Burghardt J, Kovacs G, Stackebrandt E (1999) Reclassification of the species *Kocuria erythromyxa* (Brooks and Murray 1981) as *Kocuria rosea* (Flügge 1886). Int J Syst Bacteriol 49(2):393-396.

Schumann P, Tindall BJ, Mendrock U, Kramer I, Stackebrandt E (2000) *Pelczaria aurantia* ATCC 49321T (=DSM 12801T) is a strain of *Kocuria rosea* (Flügge 1886) Stackebrandt et al. 1995. Int J Syst Evol Microbiol 50(4):1421-1424.

Semple KM, Westlake DWS (1987) Characterization of iron reducing *Alteromonas putrefaciens* strains from oil field fluids. Can J Microbiol 35:925–931.

Seo HJ, Bae SS, Lee JH, Kim SJ (2005) *Photobacterium frigidiphilum* sp. nov., a psychrophilic, lipolytic bacterium isolated from deep-sea sediments of Edison Seamount. Int J Syst Evol Microbiol 55:1661–1666.

Shade A, Hogan CS, Klimowicz AK, Linske M, McManus PS, Handelsman J (2012) Culturing captures members of the soil rare biosphere. Environ Microbiol 14(9):2247-2252.

Singh RP (2013) Studies on certain seaweed–bacterial interaction from Saurashtra coast. PhD Thesis. 161P. http:// ir.inflibnet.ac.in:8080/jspui/handle/10603/9191.

Singh RP, Reddy CRK (2014) Seaweed‐microbial interactions: key functions of seaweed ‐associated bacteria. FEMS Microbiol Ecol 88(2):213-230.

Slepecky RA (1972) Ecology of bacterial sporeformers. 297–313. H. G. Halvorson, R. Hanson, and L. L. Campbell (ed.) Spores V. American Society for Microbiology. Washington, D.C.

Slepecky RA, Hemphill HE (2006) The genus *Bacillus*—nonmedical. In The prokaryotes (pp. 530-562). Springer US.

Sober E (1983) Parsimony in Systematics: Philosophical Issues. Annu Rev Ecol Evol Syst 14:335–357.

Stabili L, Gravili C, Tredici SM, Piraino S, Tala A, Boero F, Alifano P (2008) Epibiotic Vibrio luminous bacteria isolated from some Hydrozoa and Bryozoa species. Microbial Ecol 56 (4):625-636.

Stackebrandt E, Koch C, Gvozdiak O, Schumann P (1995) Taxonomic dissection of the genus Micrococcus: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. Int J Syst Bacteriol 45(4):682-692.

Staufenberger T, Thiel V, Wiese J, Imhoff JF (2008) Phylogenetic analysis of bacteria associated with *Laminaria saccharina*. FEMS Microbiol Ecol 64: 65–77.

Steinberg PD, de-Nys R, Kjelleberg S (2002) Chemical cues for surface colonization. J Chem Ecol 28:1935–1951.

Talà A, Lenucci M, Gaballo A, Durante M, Tredici SM, Debowles DA, Pizzolante G, Marcuccio C, Carata E, Piro G, Carpita NC, Mita G, Alifano P (2013) *Sphingomonas cynarae* sp. nov., a proteobacterium that produces an unusual type of sphingan. Int J Syst Evol Microbiol 63:72–79.

Tatewaki M, Provasoli L, Pintner IJ (1993) Morphogenesis of *Monostroma oxyspermum* (KÜTZ.) Doty (Chlorophyceae) in axenic culture, especially in bialgal culture. J Phycol 19:409–416.

Thompson FL, Li Y, Gomez-Gil B, Thompson CC, Hoste B, Vandemeulebroecke K, Rupp GS, Pereira A, De Bem MM, Sorgeloos P, Swings J (2003) *Vibrio neptunius* sp. nov., *Vibrio brasiliensis* sp. nov. and *Vibrio xuii* sp. nov., isolated from the marine aquaculture environment (bivalves, fish, rotifers and shrimps). Int J Syst Evol Microbiol 53(1):245-252.

Thompson FL, Thompson CC, Naser S, Hoste B, Vandemeulebroecke K, Munn C, Bourne D, Swings J (2005) *Photobacterium rosenbergii* sp. nov. and *Enterovibrio coralii* sp. nov., vibrios associated with coral bleaching. Int J Syst Evol Microbiol 55:913–917.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Urbanczyk H, Yoshitoshi O, Hayashi T (2013) Taxonomic revision of Harveyi clade bacteria (family Vibrionaceae) based on analysis of whole genome sequences. Int J Syst Evol Microbiol 63(7):2742-2751.

Venkateswaran K, Moser DP, Dollhopf ME, Lies DP, Saffarini DA, MacGregor BJ, Ringelberg DB, White DC, Nishijima M, Sano H, Burghardt J, Stackebrandt E, Nealson KH (1999) Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. Int J Syst Bacteriol 49:705–724.

Venkateswaran K, Dohmoto N (2000) *Pseudoalteromonas peptidolytica* sp. nov., a novel marine mussel-thread-degrading bacterium isolated from the Sea of Japan. Int J Syst Evol Microbiol 50(2):565-574.

Verlaque M, Durand C, Huisman JM, Boudouresque CF, Le Parco Y (2003) On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). Eur J Phycol 38:325–339.

Vigliotta G, Tredici SM, Damiano F, Montinaro MR, Pulimeno R, di Summa R, Massardo DR, Gnoni GV, Alifano P (2005) Natural merodiploidy involving duplicated rpoB alleles affects secondary metabolism in a producer actinomycete. Mol Microbiol 55:396–412.

Vigliotta G, Nutricati E, Carata E, Tredici SM, De Stefano M, Pontieri P, Massardo DR, Prati MV, De Bellis L, Alifano P (2007) *Clonothrix fusca* Roze 1896, a filamentous, sheathed, methanotrophic gammaproteobacterium. Appl Environ Microbiol 73:3556–3565.

Yoon JH, Oh TK (2005) *Bacillus litoralis* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. Int J Syst Evol Microbiol 55(5):1945-1948.

Yoshizawa S, Tsuruya Y, Fukui Y, Sawabe T, Yokota A, Kogure K, Higgins M, Carson J, Thompson FL (2012) *Vibrio jasicida* sp. nov., a member of the Harveyi clade, isolated from marine animals (packhorse lobster, abalone and Atlantic salmon). Int J Syst Evol Microbiol 62(8):1864-1870.

Wahl M, Goecke F, Labes A, Dobretsov S, Weinberger F (2012) The second skin: ecological role of epibiotic biofilms on marine organisms. Front in microbiol 3.

3. Epibiotic culturable vibrios on the Mediterranean invasive alga *Caulerpa cylindracea*

3.1. Abstract

In aquatic environments, vibrios are among the most abundant culturable microbes found either as free-living or associated with several marine organisms. *Vibrio* illnesses due to some pathogenic vibrios are increasing worldwide so that the understanding of the mechanisms related to their spread is critically needed. In this framework, in the present study, we analysed culturable vibrios abundance and diversity associated to the surface of the green alga *Caulerpa cylindracea* (Sonder) during different algal developmental stages. Vibrios density ranged from 1.9 x 10^3 CFU mL^{-1} to 2.2 x 10^4 CFU mL^{-1}. The *Vibrio* strains isolated from *C. cylindracea* surface were assigned to: *V. tubiashii*, *V. neptunius, V. rotiferianius, V. communis*, *V. owensii*, *V. jasicida, V. harveyi, V. maritimus, V. diabolicus, V. campbellii, V. pomeroyi, V. crassostreae, V. aestivus*. None of the isolated vibros was a potential human pathogenic species but some resulted as potential pathogens of benthic organisms. Most of the observed *C. cylindracea*-*Vibrio* interactions were consistent in time. By contrast, only few ones (*V. maritimus*, *V. diabolicius*, *V. aestivus, V. pomeroyi, V. campbellii, V. crassostreae, V. harveyi*) seem to be sporadic (i.e. they were found only in certain periods of the year). This evidence is of particular importance to understand the risks of *Vibrio* infection from *C. cylindracea* in benthic assemblages. *Caulerpa cylindracea* is known to be invasive, causing environmental concern in the Mediterranean Sea during recent years. Our results suggest that the pathogenicity of the vibrios species isolated might have an effect on benthic organisms in contact with this invasive alga and might protect the alga from the colonization of its surface, thus contributing to the successful spreading of this species.

3.2. Introduction

Epibiosis is a typically aquatic phenomenon. In the marine environment, any solid, exposed, undefended surface is rapidly colonized by bacteria. The bacterial population associated with living surfaces are an important component in the development of a fouling community (Mitchell and Kirchman, 1984), and appear to be highly variable. This variability includes a wide range of epibacterial densities (from 0 to 10^8 cells per square centimeter) reported from the surfaces of algae (Johnson et al., 1991; Rosowski, 1992), sponges (Santavy and Colwell, 1990), cnidarians (Ducklow and Mitchell, 1979; Stabili et al., 2006), and bryozoans (Walls et al., 1993). Qualitatively, living marine substrates may harbor specialized microbial communities (Gil-Turnes and Fenical, 1992), often distinct from the surrounding seawater and the neighboring environments (Santavy and Colwell, 1990; Johnson et al., 1991). Based upon the great variability in microbial populations associated with living substrates, it can be suggested that some surface proprieties of organisms play a central role in the control of epibacterial distributions (Wahl et al., 1994). Many organisms seem selectively to enhance surface settlement by some bacteria and discourage colonization by others. The production of specific compounds that hinder microbial growth or inhibit microbial settlement could be responsible of these processes. Actually, settlement can be largely influenced by organic metabolites produced by the host. These metabolites may affect bacteria in several ways, ranging from the stimulation of a chemotactic response, to the inhibition of bacterial growth until to cell death (Sieburth and Conover, 1965). The degree to which marine bacteria respond to plant and invertebrate metabolites could have a profound effect upon the distributions of bacteria on living surfaces. However, to date, these responses remain poorly documented.

Seaweeds and seagrasses have dense populations of bacteria on their surfaces, although this varies considerably with species, geographic locations and climatic conditions (Goecke et al., 2013a,b). The surface of algae may play an important role in the formation of specific bacterial communities, since they are a primary source of nutrients but algal metabolites can encourage /discourage the microbiota growth (Sneed and Pohnert, 2011; Salaun et al., 2012). The algal morphology and micro-topography of surface may also influence associated bacterial biofilm, so that each seaweed represents a unique micro-ecosystem (Wahl et al., 2010). Moreover, bacteria growing on the surfaces of marine algae are in a highly competitive environment where space and nutrients are limited. Some associated bacteria produce a number of antibiotic compounds higher than that observed

in free-living bacteria isolated from marine environments (Lemos et al., 1986; Zheng et al., 2005). It has been hypothesized (Burgess et al., 1999) that competition for space and nutrients might be a selective force that may have led to the evolution of a variety of effective adaptation in several attached bacteria. Once established, specific microbes may themselves influence colonization by other fouling organisms. *Caulerpa cylindracea* (Sonder) (Belton et al., 2014), previously known as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman and Boudouresque (Verlaque et al., 2003), is a non-indigenous invasive algal species in the Mediterranean Sea (Klein and Verlaque, 2008). Observed for the first time off the coast of Libya (Nizamuddin, 1991), the introduced *C. cylindracea* has so far been reported from the most of Mediterranean countries including Italy, spreading rapidly and colonizing all available substrates and habitats. This non-indigenous alga represents a serious threat for native benthic assemblages in the Mediterranean Sea, affecting their biodiversity and habitat complexity (Piazzi et al., 2005; Thomsen et al., 2011). Consequently, *C. cylindracea* has been included in the '100 worst invaders' list for the Mediterranean Sea (Streftaris and Zenetos, 2006). Previous studies on the other invasive algae *C. taxifolia* (Meusnier et al., 2001) and *C. cylindracea* (Aires et al., 2013) have shown that an important bacterial community is associated with their thalli. These observations lead to hypothesize that the algal host obtains clear benefits from the microbial association relying on secondary metabolites produced by surface-associated bacteria as their defense against fouling. These benefits might contribute to the spreading of these algal species. In this framework, we examined the *Vibrio* diversity associated to *C. cylindracea*. Vibrios are Gram-negative curved bacteria naturally present in marine, estuarine, and freshwater, preferring to live attached on biotic and abiotic substrata than free-floating. Some members of the genus *Vibrio* have been described as the main etiological agents of diseases affecting humans and marine organisms, whereas some species are necessary for driving fundamental ecosystem processes, such as the carbon cycle and osmoregulation (Johnson, 2013).

In the present study we evaluated culturable *Vibrio* colonizing the surface of *C. cylindracea* in time: at the beginning of the vegetative phase, during the algal growth and during its maximum. We complemented culture-based with molecular methods, which, as already recently reported by Guerrero-Ferreira et al. (2013), is an excellent approach to describe the nature of bacterial communities. Recently the application of omic approaches contributed to relevant advances when the aim is to describe total bacterial diversity. However, studying organisms in pure culture allows an higher taxonomic effort (even

though limited to culturable bacteria), to address bacterial physiology and metabolism, and to assess the relationships that bacteria establish with other organisms (Anonymous, 2013). Our study was designed to assess: (1) if the culturable vibrios community varies during the algal developmental stages, and consequently (2) whether there is a specific vibrios community associated to the seaweed. The ecological implications are discussed considering the metabolic and pathogenic features of the identified vibrios and their potential role in contributing to *C. cylindracea* spreading and habitat colonization.

3.3. Materials and methods

Sampling

Caulerpa cylindracea was collected by SCUBA divers in the Marine Protected Area of Torre Guaceto (Brindisi, Italy) at shallow water (5-10 m depth) on rocky substrates (Fig. 3.1).

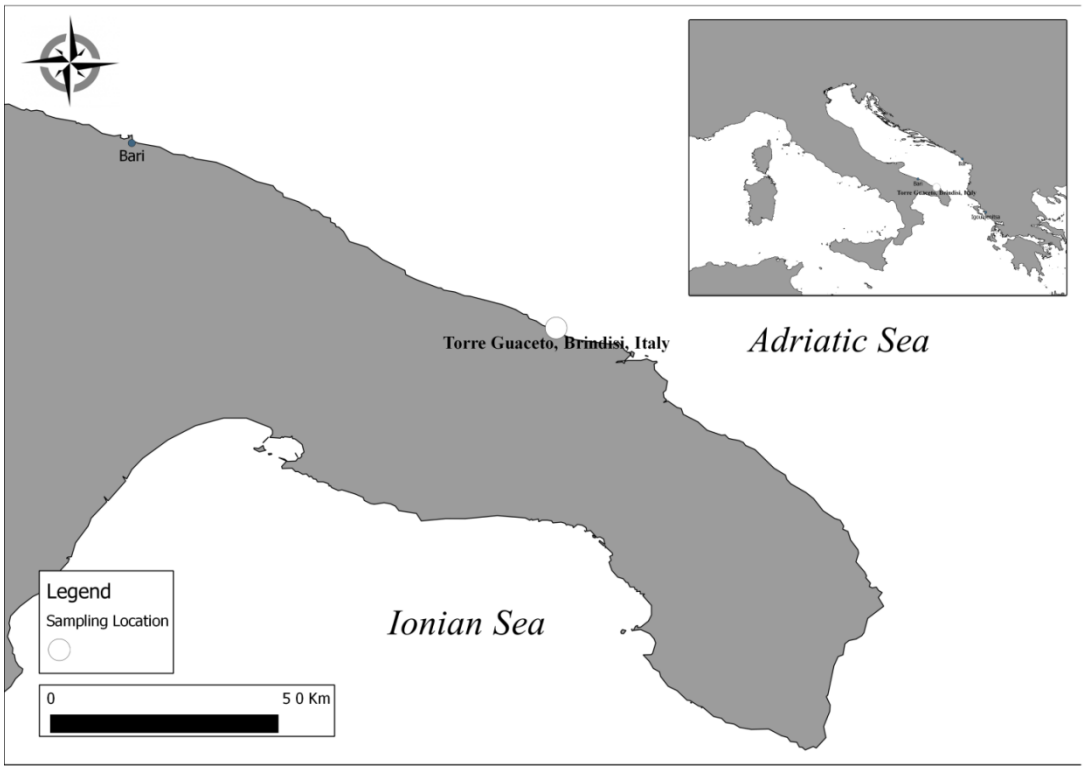


Figure 3.1. Map of the Apulian Region (Italy, Mediterranean Sea) showing the sampling point of *C. cylindracea*.

Caulerpa cylindracea shows a seasonal cycle with a period of vegetative growth approximately between June and November alternated with a period of vegetative rest (a quasi-complete withdrawal) approximately from December to May (Komatsu et al., 1997;

Piazzi et al., 2001; Riutton et al., 2005). During this vegetative rest period, despite the drop of cover and biomass, some stolons remain in the substrate, allowing a fast recolonization at the beginning of the following spring-summer. Samplings were carried out in four different times: September 2012 (time 1), May (time 2), July (time 3) and September 2013 (time 4). At each time, about 300 g of *C. cylindracea* were collected in triplicate and transferred to the laboratory under controlled temperature and processed for culturable *Vibrio* isolation and enumeration within 4 h from collection.

Vibrios Enumeration and Isolation

In the laboratory, the seaweed was washed several times in sterile seawater (0.2 μm pore filtered) to eliminate the bacteria settled on the surfaces, then suspended in sterile seawater and sonicated three times (Branson Sonifier 2200, 60 W, 47 kHz for 1 min in an ice bath) to optimize bacteria detachment from the biofilm formed on the alga. The sonication was interrupted every 30 min, and samples were shaken manually. In order to enumerate the culturable vibrios, 1 or 5 mL of each sonicated sample and appropriate decimal dilutions were plated in triplicates on to thiosulphate–citrate–bile–sucrose–salt (TCBS) agar. Incubation was carried out at 20-25 °C and 35 °C for 2 days. The incubation temperature of 35 °C was chosen to estimate the fraction of vibrios potentially pathogenic to humans. The incubation temperature of 20-25 °C was chosen since some *Vibrio* spp., such as *Vibrio anguillarum*, do not grow well at higher temperatures (Planas et al., 2006). After incubation, the colonies of presumptive vibrios were counted in accordance with the colony-forming unit (CFU) method. Mean values from three replicates were determined and expressed as CFU mL^{-1}, taking account of the dilution factor. All the suspected colony types (yellow or green) grown on TCBS agar were picked out and streaked on to Marine Agar to obtain pure cultures.

Phenotypic characterization of *Vibrio* isolates

On the bacterial isolates for phenotypic identification the following tests were performed: Gram stain, growth in TCBS agar, bioluminescence on luminescence agar (West and Colwell, 1984), oxidase and catalase assays, sensitivity to the vibriostatic agent O/129 (10 μg), and amino acid decarboxylase reaction. Growth at 0, 3, 6 and 10 % NaCl, growth

at 4 °C, 30 °C, 35 °C and 40 °C, indole production, gelatinase production, esculin hydrolysis, urease production, β-galactosidase production, nitrite production, utilization of citrate, and starch and acid production from glucose and sucrose were also determined. A further characterisation was performed by screening the utilization of various carbon sources (starch, L-arabinose, N-acetyl-glucosamine, mannose, glucose and mannitol) with the addition of NaCl to a final concentration of 2.5%.

16S rRNA Gene Sequence Analysis of Bacterial Isolates

High molecular weight genomic DNA from the different bacterial isolates was prepared according to standard procedures. Strains were grown in 100 mL nutrient broth (Difco) containing 3% NaCl with shaking at 30° C to an optical density of 0.8 at 550 nm. After centrifugation, pellets were washed with 50 mL of STE buffer [500 mM NaCl, 50 mM Tris–Cl (pH 8), 5 mM EDTA], and then resuspended in 4 mL of a solution containing 50 mM Tris–Cl (pH 8), 25% sucrose, 1 mM EDTA. Lysozyme (1 mg mL^{-1}) treatment was carried out at 0 °C for 10 min, then EDTA was added to a final concentration of 40 mM, and samples were incubated at 0 °C for 10 min. Proteinase K (100 mg mL^{-1}) treatment was performed for 2 h at 65 °C after addition of sodium dodecyl sulfate (SDS) to a final concentration of 1%. Nucleic acids were extracted by phenol–chloroform/isoamylic alcohol (24:1) extraction according to standard procedures (Sambrook and Russell, 2001), and 15 μg mL^{-1} ribonuclease A were used to remove RNA. After phenol–chloroform/isoamylic alcohol (24:1) extraction and ethanol precipitation, high molecular weight DNA was collected by spooling using Shepherd's crooks (Sambrook and Russell, 2001).

The 16S rRNA-encoding genes were amplified using the Eubacterial-specific primers 16SEB20-43-F (5′-TGGCTCAGATTGAACGCTGGCGG-3′) and 16SEB1488-R (5′-TACCTTGTTACGACTTCACC-3′) (Vigliotta et al., 2007; Talà et al., 2013). The two primers were designed to amplify a 1488 bp-long DNA fragment (from nucleotide 20 to nucleotide 1507 in the *Escherichia coli* 16S rRNA gene). PCR reactions were performed as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 55 °C and extension at 72 °C for 1-2 min, and the final elongation step at 72 °C for 5 min. They were carried out in a Perkin-Elmer Cetus DNAThermal Cycler 2400. PCR products were isolated through 1%agarose gels in 1X TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0), recovered using the Qiaex

II Gel extraction kit (Qiagen) and finally sequenced as a service by MWG Biotech Custom Sequencing Service (Germany). The sequences of all isolates were compared with those of closely related reference strains using the EzTaxon-e server (Kim et al., 2012). Multiple sequence alignments were performed with CLUSTAL W (Thompson et al., 1994) at the Kyoto University Bioinformatic Center (http://www.genome.jp/tools/clustalw/) using the following default settings. The CLUSTAL W output file was used to construct evolutionary trees with the SeaView software (Galtier et al., 1996; Gouy et al., 2010) in accordance with the neighbour-joining (Saitou and Nei, 1987), maximum-parsimony (Sober, 1983) and maximum-likelihood (Felsenstein, 1981) methods. Evolutionary distances were calculated with the neighbor-joining method in accordance with the algorithm of Kimura's two parameter model (Kimura, 1980). Tree robustness was assessed by bootstrap resampling (1,000 replicates each) (Brown, 1994). The nucleotide sequence of almost the entire 16S rRNA-encoding gene of *Vibrio* sp. isolates was deposited at GenBank with the following accession numbers: KM014009 (CR-I-1), KM014010 (CR-I-2), KM014011 (CR-I-3), KM014012 (CR-I-4), KM014013 (CR-I-5), KM014014 (CR-I-6), KM014015 (CR-I-7), KM014016 (CR-I-8), KM014017 (CR-II-9), KM014018 (CR-II-10), KM014019 (CR-II-11), KM014020 (CR-II-12), KM014021 (CR-II-13), KM014022 (CR-II-14), KM014023 (CR-II-15), KM014024 (CR-II-16), KM014025 (CR-II-17), KM014026 (CR-III-18), KM014027 (CR-III-19), KM014028 (CR-III-20), KM014029 (CR-III-21), KM014030 (CR-III-22), KM014031 (CR-III-23), KM014032 (CR-III-24), KM014033 (CR-IV-25), KM014034 (CR-IV-26), KM014035 (CR-IV-27), KM014036 (CR-IV-28), KM014037 (CR-IV-29), KM014038 (CR-IV-30), KM014039 (CR-IV-31), KM014040 (CR-IV-32), KM014041 (CR-IV-33) and KM014042 (CR-IV-34).

3.4. Results

Microbiological Analyses and Isolation of *Vibrio* sp. in Pure Cultures

Vibrios densities on the surface of *C. cylindracea* in the four sampling times are reported in Fig. 3.2. In particular, in time 1, on average, vibrios accounted for 1.2 x 10^4 CFU mL^{-1}, in time 2 their density reached 1.9 x 10^3 CFU mL^{-1}, 1.8 x 10^4 CFU mL^{-1} in time 3 and 2.2 x 10^4 CFU mL^{-1} in time 4.

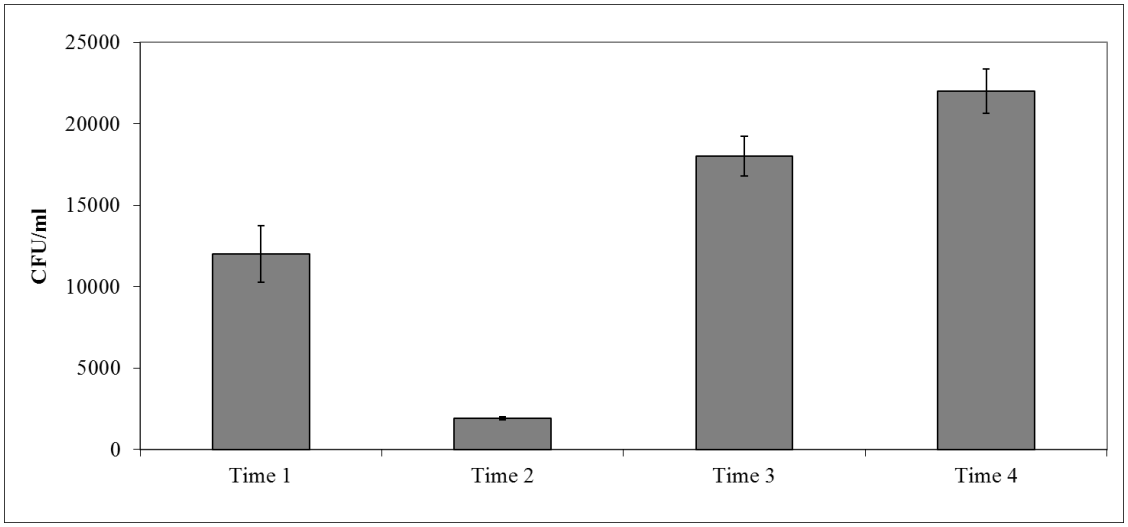


Figure 3.2. Mean abundance and relative standard deviation of vibrios densities on *C. cylindracea* collected in the four sampling times.

Identification of *Vibrio* sp. Isolates from *C. cylindracea* surface

For each sampling time, a total of fifty *Vibrio* sp. isolates were preliminarily analyzed and grouped into nine types, based on their cultural and biochemical properties for the first and second sampling time, seven types for the third sampling time and ten types for the fourth time. Representative isolates from each of the type groups were characterized by nucleotide sequencing of almost the entire 16S-rRNA-encoding genes and further biochemical tests. These isolates were designated with the abbreviation CR, indicating the source of the isolation, followed by two numbers one referred to the sampling time and indicated by a roman numeral and one referred to the sequential number.

Table 3.1. *Vibrio* diversity on *C. cylindracea* surface collected in the four sampling times

Vibrio species	I	II	III	IV
V. aestivus	-	-	-	+
V. campbellii	-	-	-	+
V. communis	+	+	+	+
V. crassostreae	-	+	-	-
V. diabolicus	+	+	-	-
V. harveyi	-	-	+	+
V. jasicida	+	+	+	+
V. maritimus	+	-	-	+
V. neptunius	+	+	+	+
V. owensii	+	+	+	+
V. pomeroyi	-	+	-	-
V. rotiferianus	+	+	+	+
V. tubiashi	+	+	+	+

The *Vibrio* strains isolated from *C. cylindracea* surface were: *V. tubiashii*, *V. neptunius, V. rotiferianius, V. communis*, *V. owensii*, *V. jasicida, V. harveyi, V. maritimus, V. diabolicus, V. pomeroyi, V. crassostreae, V. campbellii* and *V. aestivus* (Fig. 3.3 a-b).

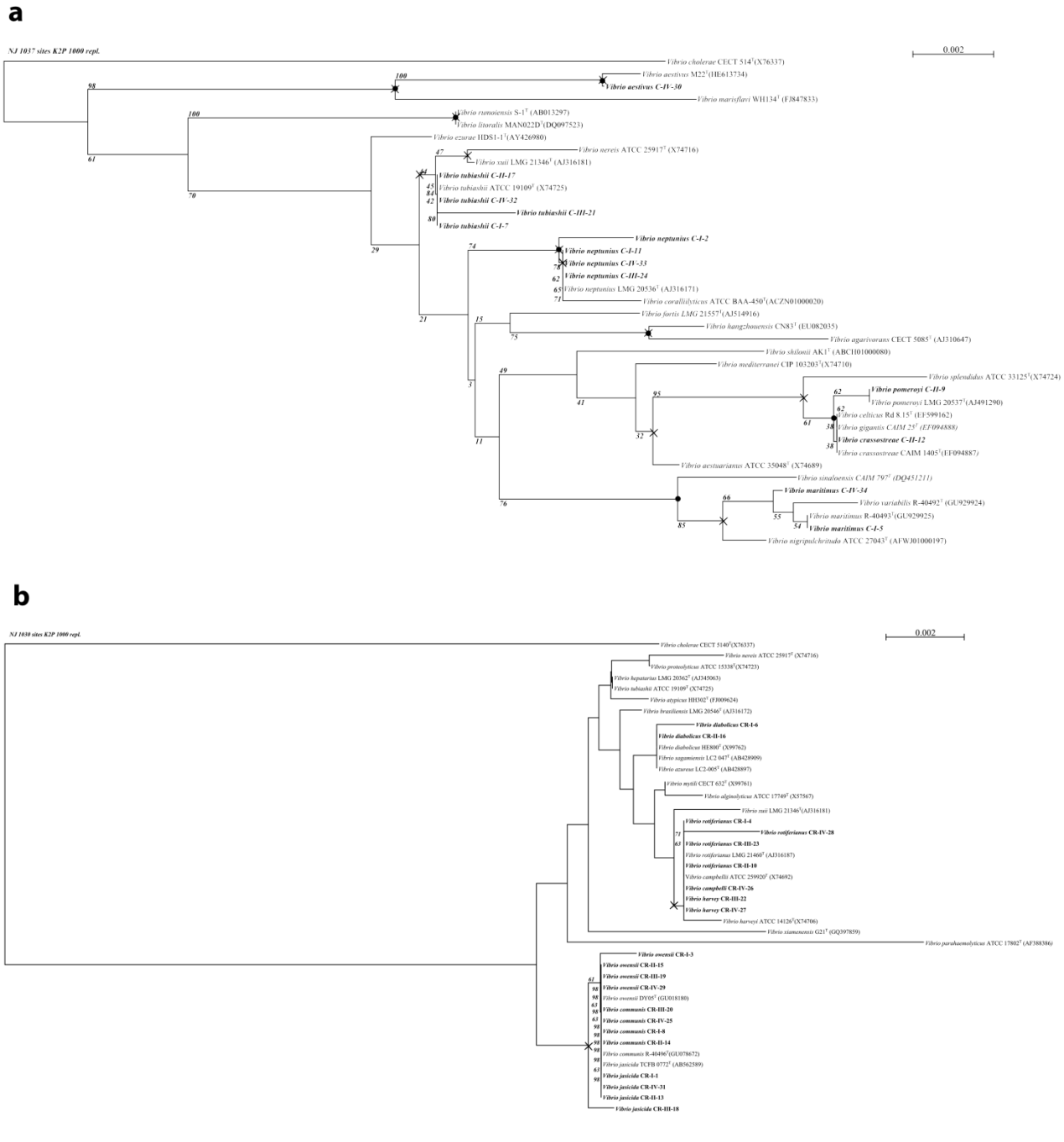


Figure 3.3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing positions of (**a)** *Vibrio aestivus*, *V. tubiashii*, *V. neptunius*, *V. pomeroyi*, *V. crassostreae* and *V. maritimus* and (**b**) *Vibrio diabolicus*, *V. rotiferianus*, *V. campbellii*, *V. harveyi*, *V. owensii*, *V. communis* and *V. jasicida* from *C. cylindracea* surface (in bold) with respect to closely-related species. Bootstrap values (expressed as percentages of 1,000 replicates) of >60 % are shown at branch point. Filled circles and "X" indicate that corresponding nodes were also recovered in phylogenetic trees constructed with maximum parsimony and maximum-likelihood algorithms respectively. *Vibrio cholerae* CECT 514T was used as outgroup.

In Table 3.1 the presence of the culturable isolated vibrios in the four sampling times is reported. *Vibrio tubiashii* was isolated in all the sampling times. In particular EzTaxon-e analysis of the 16S rRNA gene sequence of *Vibrio* CR-I-7, CR-II-17, CR-III-21 and CR-IV-32 indicated that the closest relative of these strains (99,75%, 99,85%, 99,85% and 100% identity respectively) was *Vibrio tubiashii* ATCC 19109^T (Hada et al., 1984). In the neighbour-joining tree, CR-I-7, CR-II-17, CR-III-21 and CR-IV-32 branched together with the aforesaid reference strain (Fig. 3.3a). The relationship between CR-I-7, CR-II-17, CR-III-21 and CR-IV-32 and *Vibrio tubiashii* ATCC 19109^T (Hada et al., 1984) was also maintained in trees constructed with the maximum-parsimony and maximum-likelihood algorithms. The assignment of these bacterial isolates to the above-mentioned species was also consistent with the results of morphological, cultural, and biochemical tests (Table S1 of Electronic Supplementary Material), in accordance with the schemes proposed by Hada et al. (1984) and Noguerola and Blanch (2008).

Vibrio neptunius, Vibrio rotiferianius, Vibrio communis, *Vibrio owensii* and *Vibrio jasicida* were also recorded in all the sampling times. In particular, the 16S rRNA gene sequence of *Vibrio* sp. CR-I-2, CR-II-11, CR-III-24 and CR-IV-33 (99,77%, 100%, 100% and 100% sequence identity respectively) appeared to be closely related to that of the reference strain *V. neptunius* LMG 20536 ^{T} (Thompson et al., 2003a) isolated from the marine aquaculture environment (bivalves, fish, rotifers and shrimps). These isolates clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees together with *V. neptunius* LMG 20536 ^{T} (Thompson et al., 2003a) and *Vibrio coralliilyticus* ATCC BAA-450 ^{T} (Ben-Haim et al., 2003). Phenotypic analyses (Table S2) confirmed that *Vibri*o sp. CR-I-2, CR-II-11, CR-III-24 and CR-IV-33 was closely related to *V. neptunius* according to Thompson et al. (2003a).

The 16S rRNA gene sequence of *Vibrio* sp. CR-I-4, CR-II-10, CR-III-23 and CR-IV-28 seemed to be close to that of the reference strain *V. rotiferianus* LMG 21460 ^{T} (Gomez-Gil et al., 2003), isolated from cultures of the rotifer *Brachionus plicatilis* (100%, 100%, 99,91% and 99,86% identity respectively). These isolates clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees together with *V. rotiferianus* LMG 21460T (Gomez-Gil et al., 2003) and *Vibrio campbellii* ATCC 25920^T (Baumann et al., 1980) and *Vibrio harveyi* ATCC 14126 ^{T} (Johnson and Shunk, 1936; Hendrie et al., 1970; Baumann et al., 1980). Phenotypic analyses confirmed that *Vibri*o sp. CR-I-4, CR-II-10, CR-III-23 and CR-IV-28 was closely related to *V. rotiferianus* LMG 21460 ^{T} (Gomez-Gil et al., 2003). The assignment of the bacterial isolate to *V.*

rotiferrianus was confirmed by the results of morphological, cultural and biochemical tests (Table S2).

EzTaxon-e analysis of the 16S rRNA gene sequence of *Vibrio* sp. CR-I-8, CR-I-14, CR-III-20 and CR-IV-25 revealed an high identity (100%, 100%, 99,93% and 100%, respectevely) with the 16S rRNA gene sequence of *V. communis* type strain R-40496^T (Chimetto et al., 2011a) and *V. owensii* DY05^T (Cano-Gomez et al., 2010). In the neighbour-joining, maximum-parsimony and maximum-likelihood trees they grouped together with *V. communis* R-40496^T (Chimetto et al., 2011a), *V. owensii* DY05^T (Cano-Gomez et al., 2010) and *V. jasicida* TCFB 0772^T (Yoshizawa et al., 2012) (Fig. 3.3b). The assignment of the bacterial isolates to *V. communis* was confirmed by the results of morphological, cultural and biochemical tests (Table S3).

The 16S rRNA gene sequences of *Vibrio* sp. CR-I-3 CR-II-15, CR-III-19 and CR-IV-29 isolates showed an identity of 99,85%, 100% 99,86% and 99.93% with the homologous gene sequence of *V. owensii* DY05^T (Cano-Gomez et al., 2010), isolated from cultured crustaceans in Australia. In the neighbour-joining, maximum-parsimony and maximum-likelihood trees the four isolates clustered together with the reference strains *Vibrio owensii* DY05^T (Cano-Gomez et al., 2010), *Vibrio communis* R-40496^T (Chimetto et al., 2011a) and *V. jasicida* TCFB 0772^T (Yoshizawa et al., 2012). The assignment of the four bacterial isolates to the above-mentioned species was supported by the results of cultural and biochemical assays (Table S3).

EzTaxon-e analysis of the 16S rRNA gene sequence of *Vibrio* sp. CR-I-1, CR-II-13, CR-III-18 and CR-IV-31 demonstrated an identity of 100% with the homologous sequence of *V. jasicida* type strain TCFB 0772^T (Yoshizawa et al., 2012). In the neighbour-joining, maximum-parsimony and maximum-likelihood phylogenetic trees, these isolates branched with *V. jasicida* type strain TCFB 0772^T (Yoshizawa et al., 2012), *V. owensii* DY05 ^{T} (Cano-Gomez et al., 2010) and *Vibrio communis* R-40496T (Chimetto et al., 2011a). The results of cultural and biochemical assays (Table S3) confirmed the result of the phylogenetic analysis indicating that *Vibrio* sp. CR-I-1, CR-II-13, and CR-IV-31 may belong to the species *V. jasicida* (Yoshizawa et al., 2012).

Vibrio sp. CR-III-22 CR-IV-27 recorded in sampling times 3 and 4 showed an identity of 99,85% and 99,79% respectively with the *V. harveyi* type strain ATCC 14126^T (Johnson and Shunk, 1936; Hendrie et al., 1970; Baumann et al., 1980). These isolates clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees together with *V. harveyi* ATCC 14126^T (Johnson and Shunk, 1936; Hendrie et al., 1970; Baumann et al.,

1980), *V campbellii* ATCC 25920^T (Baumann et al., 1980) and *Vibrio rotiferianus* LMG 21460T (Gomez-Gil et al., 2003). The results of cultural and biochemical assays (Table S4) confirmed the result of the phylogenetic analysis indicating that the isolated vibrios species belong *to V.harveyi* (Noguerola and Blanch, 2008). In the sampling time 4 the 16S rRNA gene sequences of *Vibrio* sp. CR-IV-26 showed an identity of 99,86% with the homologous gene sequence of *V. campbellii* ATCC 25920^T (Baumann et al., 1980), isolated from diseased farm-shrimps (Haldar et al., 2011). This isolate clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees together with *V. campbellii* ATCC 25920^T (Baumann et al., 1980), *V. rotiferianus* LMG 21460^T (Gomez-Gil et al., 2003) and *V. harveyi* ATCC 14126^T (Johnson and Shunk, 1936; Hendrie et al., 1970; Baumann et al., 1980). The results of cultural and biochemical assays (Table S4) confirmed the result of the phylogenetic analysis indicating that *Vibrio* sp. CR-IV-26 may belong to the species *V. campbellii* (Noguerola and Blanch, 2008).

Vibrio maritimus was identified only in sampling times 1 and 4. In particular *Vibrio* sp. CR-I-5 and CR-IV-34 demonstrated an identity of 99.91% and 99.86% with the homologous sequence of *V. maritimus* type strain R40493^T (Chimetto et al., 2011b). These isolates clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees together with *V. maritimus* R40493^T (Chimetto et al., 2011b) and *Vibrio variabilis* R40492^T (Chimetto et al., 2011b). The assignment of these bacterial isolates to the above-mentioned species was supported by the results of cultural and biochemical assays (Table S5).

Vibrio diabolicus was recognized sampling times 1and 2. The isolates CR-I-6 CR-II-16 showed an identity of 99.92% and 100% respectively with *V. diabolicus* HE800^T (Raguenes et al., 1997). These isolates clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees together with *Vibrio sagamiensis* LC2 047 (Yoshizawa et al., 2010) and *Vibrio azureus* LC2 005^T (Yoshizawa et al., 2009). The assignment of these bacterial isolates to the above-mentioned species was supported by the results of cultural and biochemical assays (Table S5).

Some *Vibrio* species were isolated only one time during the study period. In particular in the second sampling time the 16S rRNA gene sequence of *Vibrio* sp. CR-II-9 appeared to be closely related to that of the reference strain *Vibrio pomeroyi* LMG 20537^T (Thompson et al., 2003b) (100 % identity), in the neighbour-joining, maximum-parsimony and maximum-likelihood trees confirmed these results. The 16S rRNA gene sequence of *Vibrio* sp.CR-II-12 showed an identity of 100%to the reference strain *Vibrio crassostreae*

CAIM 1405^T (Faury et al., 2004). This isolate clustered in the neighbour-joining, maximum-parsimony and maximum-likelihood trees together with *V. crassostreae* CAIM 1405^T (Faury et al., 2004) and *Vibrio gigantis* CAIM 25^T (Le Roux et al., 2005) and *Vibrio celticus* Rd 8.15^T (Beaz-Hidalgo et al., 2010). The results of cultural and biochemical assays (Table S6) confirmed the result of the phylogenetic analysis indicating that *Vibrio* sp. CR-II-9 and CR II-12 may belong to the species *V. pomeroy and V. crassostreae* respectively (Noguerola and Blanch, 2008).

In the sampling time 4 the 16S rRNA gene sequences of *Vibrio* sp. CR-IV-30 showed an identity of 99,93% with the homologous gene sequence of *Vibrio aestivus* M22^T (Lucena et al., 2012), isolated from seawater collected off a beach on the East coast of Spain. In the neighbour-joining, maximum-parsimony and maximum-likelihood trees confirmed these results. The assignment of this bacterial isolate to the above-mentioned species was supported by the results of cultural and biochemical assays (Table S7).

3.5. Discussion

In present study we investigated, for the first time, the diversity of culturable vibrios associated to the surface of the Mediterranean invasive alga *C. cylindracea* surface throughout a year, by complementing culture-based with molecular methods. Algal-bacterial associations are fundamental to the biology and the ecology of both organisms, but the complexity of these associations remains still largely unknown as well as how bacterial-algal associations influence metabolic and developmental features of the hosts. In addition, in most cases, bacteria involved are not identified. Traditionally, the first step in investigations of microbial associations and identification of the organisms involved is to isolate them into pure culture. Here, the culturable vibrios associated to the surface of *C. cylindracea* were identified allowing us to describe seaweed-bacterial associations still largely neglected. Depending on the bacterial biofilm composition, the seaweed may be protected from detrimental colonization (Goecke et al., 2010; Nasrolahi et al., 2012) or fall into severe disease (Goecke et al., 2010; Vairappan et al., 2010). Therefore, knowing the diversity of vibrios associated to *C. cylindracea* is of fundamental importance to understand their role in protecting the seaweed from colonization as well as the role of the alga as reservoir for several *Vibrio* species.

From our data some interesting issues can be inferred:

Thirteen *Vibrio* species were identified from *C. cylindracea* surface and assigned to known type strains not including human pathogenic species. However, among these strains, potential pathogens of benthic organisms, some of which belonging to the Harveyi clade, have been found.

- Members of the *V. harveyi* clade are major pathogens of many aquatic organisms including vertebrates and invertebrates (Urbanczyk et al., 2013). In particular, strains CR-I-4, CR-II-10, CR-III-23 and CR-IV-28 isolated from *C. cylindracea* surface belong to the species *V. rotiferianus*. This species was originally isolated from cultures of the rotifer *Brachionus plicatilis*, which serve as important nutrients for fish and crustaceans in aquaculture industries (Gomez-Gil et al., 2003). *V. rotiferianus* is closely related to *V. campbellii* and *V. harveyi.* These three last *Vibrio* species, forming the most recent subclade of speciation within the Harveyi clade (Pascual et al., 2009). *Vibrio rotiferianus* is a marine pathogen able to bring about disease in various aquatic organisms. In our study this *Vibrio* species was recorded in all the four sampling times and *C. cylindracea*, was never affected by disease, highlighting the importance of exploring the relationships that the above-mentioned pathogenic *Vibrio* species have established with the examined alga. In this framework, it is noteworthy that *V. rotiferianus* has been already isolated from the brown alga *Delesseria sanguinea* (Goecke et al., 2013a). This bacterial strain associated with the brown macroalga interestingly exhibited antibacterial activity suggesting a defensive role by inhibiting the growth of other pathogens that might affect the macroalga (Goecke et al., 2013a,b). High antimicrobial productivity against microbial competitors and algal pathogens sustains the hypothesis that complex biochemical interactions characterise the associations between bacteria and macroalgae suggesting that these bacteria represent a source of antibacterial metabolites. Further studies will be undertaken to confirm this hypothesis in the case of *V. rotiferianus* associated to *C. cylindracea*. Stains CR-I-8, CR-II-14, CR-III-20 and CR-IV-25 clustered together *V. communis*. *V. communis* was isolated in 2011 from the marine corals *Mussismilia hispida* and *Phyllogorgia dilatata*, the zoanthids *Palythoa caribaeorum* and *Palythoa variabilis* and the Pacific white shrimp *Litopenaeus vannamei* (Chimetto et al., 2011a). Therefore, the data obtained in our study are of critical importance since they are the first documented case of *V. communis* associated to a seaweed. Thus, the association of this *Vibrio* species and the surface of C. *cylindracea* needs to be elucidated. *Vibrio harveyi* was isolated only in sampling times 3 and 4. This species is responsible for vast mass mortalities in shrimp farms (Owens and Busico-Salcedo, 2006) and also hitting pearl oysters, fish, seahorses,

and lobsters (Vidgen et al., 2006). However, *V. harveyi* is also found in mutualistic association with the hydrozoan *Aglaophenia octodonta* (Stabili et al., 2006) and fish light organs (Dunlap et al., 2008). This *Vibrio* species was found on the surface of healthy *C. cylindracea* and thus presumably, similarly to the above described association it is not pathogenic also to this algal species. In the present study, *Vibrio jasicida*, another member of the Harveyi clade, was isolated from *C. cylindracea* surface throughout the year (CR-I-1, CR-II-13,CR-III-18 and CR-IV-31). This species has been already isolated from marine invertebrates and vertebrates (packhorse lobster, abalone and Atlantic salmon) between 1994 and 2002 (Yoshizawa et al., 2012). Based on both 16S gene sequences and cultural and biochemical data, strains CR-I-3 CR-II-15, CR-III-19 and CR-IV-29 were assigned to the species *V. owensii*, an invertebrate pathogen recorded throughout the year on the surface of *C. cylindracea*. This species is one of the most pathogenic vibrios and was isolated for the first time from diseased cultured crustaceans *Panulirus ornatus* and *Penaeus monodon* in Australia and has been recently recognized as a pathogen of marine-reared crustaceans (Cano-Gomez et al., 2010; Goulden et al., 2012).

- Strains CR-I-7, CR-II-17, CR-III-21 and CR-IV-32 were assigned to the species *Vibrio tubiashii*. Also this *Vibrio* was found associated to *C. cylindracea* surface throughout the year. This species was first recognized more than 40 years ago as a pathogen of several bivalve larvae (Tubiash et al., 1965; Tubiash et al., 1970). The disease was initially named bacillary necrosis, and the causative bacterium later nominated as *V. tubiashii* by Hada et al. (1984). Although *V. tubiashii* was originally found to produce bacillary necrosis in larval and juvenile mollusks (Tubiash et al., 1965;Tubiash et al., 1970; Baumann and Schubert, 1984; Hada et al., 1984), following studies have highlighted that it can also bring to diarrhoea in suckling mice (Delston et al., 2003). Furthermore, *V. tubiashii* could also be a finfish pathogen, due to its capacity to cause death in fish (Austin et al., 2005). Since we found *V. tubiashii* associated to *C. cylindracea* in all sampling times, further studies are needed to clarify the nature of such association.

- In the phylogenetic trees, CR-I-2, CR-II-11, CR-III-24 and CR-IV-33 clustered together with *V. neptunius.* This species has been already isolated from larvae of the bivalve *Nodipecten nodosus* in the south of Brazil (Thompson et al., 2003a) and was recognized as a molluscan pathogen (Prado et al., 2005). This species is highly related to the described coral-pathogenic species *V. coralliilyticus* as well as to *V. brasiliensis* and *V. xuii.*

- Importantly, only few of the isolated vibrios (*V.campbellii, V. aestivus, V. pomeroyi* and *V. crassostreae*) have a sporadic presence on the algal surface (i.e. they were found only in one time) whilst most of the isolates were found in all the four sampling times. The association of these last *Vibrio* species and the surface of *C. cylindracea* needs to be elucidated in order also to ascertain whether, according to Wahl et al. (2012), vibrios might control the host's further foulers, consumers or pathogens. Thus, as already stated by Singh and Reddy (2014) there remains much to study and understand regarding the detailed mechanisms and functional aspects of seaweed–bacterial interactions that can be better illustrated with the integration of ecological, microbiological and biochemical studies. Last but not least, since some of the bacteria exclusively associated to macroalgae interestingly often exhibit antibacterial activity, exploring *Vibrio* species isolated from *C. cylindracea* surface could produce interesting results for applicative purposes including biotechnologies.

3.6. References

Aires T, Serrão EA, Kendrick G, Duarte CM, Arnaud-Haond S (2013) Invasion Is a Community Affair: Clandestine Followers in the Bacterial Community Associated to Green Algae, *Caulerpa racemosa*, Track the Invasion Source. PLoS ONE 8(7):e68429.

Anonymous (2013) The cultural revolution. Nat Rev Microbiol 11:1–1.

Austin B, Austin D, Sutherland R, Thompson F, Swings J (2005) Pathogenicity of vibrios to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and *Artemia nauplii*. Environ Microbiol 7:1488–1495.

Baumann P, Baumann L, Bang SS, Woolkalis MJ (1980) Reevaluation of the taxonomy of *Vibrio*, *Beneckea*, and *Photobacterium*: abolition of the genus *Beneckea*. Curr Microbiol 4(3):127-132.

Baumann P, Schubert RHW (1984) Family II. Vibrionaceae, p. 516–544. In N. R. Krig and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, 3rd ed. Williams and Wilkins, Baltimore, MD.

Beaz-Hidalgo R, Diéguez AL, Cleenwerck I, Balboa S, Doce A, de Vos P, Romalde JL (2010) *Vibrio celticus* sp. nov., a new *Vibrio* species belonging to the Splendidus clade with pathogenic potential for clams. Syst Appl Microbiol 33(6):311-315.

Belton GS, Prud'homme van Reine, WF, Huisman JM, Draisma SGA, Gurgel CFD (2014) Resolving phenotypic plasticity and species designation in the morphology challenging *Caulerpa racemosa-peltata* complex (Caulerpaceae, Chlorophyta). J Phycol 50(1):32-54.

Ben-Haim Y, Thompson FL, Thompson CC, Cnockaert M, Hoste B, Swings J, Rosenberg E (2003) *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. Int J of Syst Evol Micr 53:309–315.

Brown JK (1994) Bootstrap hypothesis tests for evolutionary trees and other dendrograms. Proc Natl Acad Sci USA 91:12293–12297.

Burgess JG, Jordan EM, Bregu M, Mearns-Spragg A, Boyd KG (1999) Microbial antagonism: a neglected avenue of natural products research. J Biotechnol 70:27-32.

Cano-Gómez A, Goulden EF, Owens L, Høj L (2010) *Vibrio owensii* sp. nov., isolated from cultured crustaceans in Australia. FEMS Microbiol Lett 302(2):175-181.

Chimetto LA, Cleenwerck I, Alves N, Silva BS, Brocchi M, Willems A, De Vos P, Thompson FL (2011a) *Vibrio communis* sp. nov., isolated from the marine animals *Mussismilia hispida*, *Phyllogorgia dilatata*, *Palythoa caribaeorum*, *Palythoa variabilis* and *Litopenaeus vannamei*. Int J Syst Evol Micr 61(2):362-368.

Chimetto LA, Cleenwerck I, Moreira AP, Brocchi M, Willems A, De Vos P, Thompson FL (2011b) *Vibrio variabilis* sp. nov. and *Vibrio maritimus* sp. nov., isolated from *Palythoa caribaeorum*. Int J Syst Evol Microbiol 61(12): 3009-3015.

Delston RB, Kothary MH, Shangraw KA, Tall BD (2003) Isolation and characterization of zinc-containing metalloprotease expressed by *Vibrio tubiashii*. Can J Microbiol 49:525–529.

Ducklow HW, Mitchell R (1979) Bacterial populations and adaptations in the mucus layers on living corals. Limnol Oceanogr 24:4715–4725.

Dunlap PV, Davis KM, Tomiyama S, Fujino M, Fukui A (2008) Developmental and microbiological analysis of the inception of bioluminescent symbiosis in the marine fish *Nuchequula nuchalis* (Perciformes: Leiognathidae). Appl Environ Microbiol 74:7471–7481.

Faury N, Saulnier D, Thompson FL, Gay M, Swings J, Le Roux F (2004) *Vibrio crassostreae* sp. nov., isolated from the haemolymph of oysters (*Crassostrea gigas*). Int J Syst Evol Microbiol 54(6):2137-2140.

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376.

Galtier N, Gouy M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12: 543–548.

Gil-Turnes MS, Fenical W (1992) Embryos of *Homarus americanus* are protected by epibiotic bacteria. Biol Bull 182:105–108.

Goecke F, Labes A, Wiese J, Imhoff JF (2010) Chemical interactions between marine macroalgae and bacteria. Mar Ecol-Prog Ser 409:267–300.

Goecke F, Labes A, Wiese J, Imhoff JF (2013a) Phylogenetic analysis and antibiotic activity of bacteria isolated from the surface of two co-occurring macroalgae from the Baltic Sea. Eur J Phycol 48(1):47-60.

Goecke F, Thiel V, Wiese J, Labes A, Imhoff JF (2013b) Algae as an important environment for bacteria – phylogenetic relationships among new bacterial species isolated from algae. Phycologia 52:14–24.

Gomez-Gil B, Thompson FL, Thompson CC, Swings J (2003) *Vibrio rotiferianus* sp. nov., isolated from cultures of the rotifer *Brachionus plicatilis*. Int J Syst Evol Microbiol 53:239–243.

Goulden EF, Hall MR, Bourne DG, Pereg LL, Høj L (2012) Pathogenicity and infection cycle of *Vibrio owensii* in larviculture of the ornate spiny lobster (*Panulirus ornatus*). Appl Environ Microbiol 78(8):2841-2849.

Gouy M, Guindon S, Gascuel O (2010) SeaView Version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27(2):221-224.

Guerrero-Ferreira R, Gorman C, Chavez AA, Willie S, Nishiguchi MK (2013) Characterization of the bacterial diversity in Indo-West Pacific loliginid and sepiolid squid light organs. Microb Ecol 65(1):214-226.

Hada HS, West PA, Lee JV, Stemmler J, Colwell RR (1984) *Vibrio tubiashii* sp. nov., a Pathogen of Bivalve Mollusks. Int J Syst Bacteriol 34(1):1-4.

Haldar S, Chatterjee S, Sugimoto N, Das S, Chowdhury N, Hinenoya A, Asakura M, Yamasaki S (2011) Identification of *Vibrio campbellii* isolated from diseased farm-shrimps from south India and establishment of its pathogenic potential in an Artemia model. Microbiology 157(1):179-188.

Hendrie MS, Hodgkiss W, Shewan JM (1970) The Identification, Taxonomy and Classification of Luminous Bacteria. J Gen Microbiol 64(2):151-169.

Johnson CN (2013) Fitness factors in vibrios: a mini-review. Microb Ecol 65(4):826-851.

Johnson CR, Muir DG, Reysenbach AL (1991) Characteristic bacteria associated with surfaces of coralline algae: a hypothesis for bacterial induction of marine invertebrate larvae. Mar Ecol-Prog Ser 74:281–294.

Johnson FH, Shunk IV (1936) An interesting new species of luminous bacteria. J Bacteriol 31(6):585.

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120.

Klein J, Verlaque M (2008) The *Caulerpa racemosa* invasion: a critical review. Mar Pollut Bull 56:205–225.

Komatsu T, Meinesz A, Buckles D (1997) Temperature and light responses of alga *Caulerpa taxifolia* introduced into the Mediterranean Sea. Mar Ecol Prog Ser 146:145–153.

Le Roux F, Goubet A, Thompson FL, Faury N, Gay M, Swings J, Saulnier D (2005) *Vibrio gigantis* sp. nov., isolated from the haemolymph of cultured oysters (*Crassostrea gigas*). Int J Syst Evol Microbiol 55(6):2251-2255.

Lemos ML, Toranzo AE, Barja LJ (1986) Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. Microb Ecol 11:149-163.

Lucena T, Ruvira MA, Arahal DR, Macián MC, Pujalte MJ (2012) *Vibrio aestivus* sp. nov. and *Vibrio quintilis* sp. nov., related to Marisflavi and Gazogenes clades, respectively. Syst Appl Microbiol 35(7):427-431.

Meusnier I, Olsen JL, Stam WT, Destombe C, Valero M (2001) Phylogenetic analyses of *Caulerpa taxifolia* (Chlorophyta) and of its associated bacterial microflora provide clues to the origin to the Mediterranean introduction. Mol Ecol 10:931–946.

Mitchell R, Kirchman D (1984) The microbial ecology of marine surfaces. In: Costlow, J. D., Tipper, R. C. (eds.) Marine biodeterioration: an interdsciplinary study. E. & F. N. Spon Ltd, London. 49-58.

Nasrolahi A, Stratil SB, Jacob KJ, Wahl M (2012) A protective coat of microorganisms o macroalgae: Inhibitory effects of bacterial microfilms and epibiotic microbial assemblages on barnacle attachment. FEMS Microbiol Ecol 81:583–595.

Nizamuddin M (1991) The Green Marine Algae of Libya. Elga, Bern. Occhipinti-Ambrogi, A., Savini, D., 2003. Biological invasions as a component of global change in stressed marine ecosystems. Mar Pollut Bull 46:542–551.

Noguerola I, Blanch AR (2008) Identification of *Vibrio* spp. with a set of dichotomous keys. J Appl Microbiol 105:175-185.

Owens L, Busico-Salcedo N (2006) *Vibrio harveyi*: pretty problems in paradise. In: Thompson FL (ed) The biology of vibrios. ASM Press, Washington, D.C. 266–280.

Pascual J, Macian MC, Arahal DR, Garay E, Pujalte MJ (2009) Multilocus sequence analysis of the central clade of the genus *Vibrio* by using 16S rRNA, recA, pyrH, rpoD, gyrB, rctB, and toxR genes. Int J Syst Evol Microbiol. 60(1):154-165.

Piazzi L, Ceccherelli G, Cinelli F (2001) Threat to macroalgal diversity: effects of the introduced green alga *Caulerpa racemosa* in the Mediterranean. Mar Ecol-Progr Ser 210:161–165.

Piazzi L, Meinesz A, Verlaque M, Akçali B, Antolic B, Argyrou M, Balata D, Ballesteros E, Calvo S, Cinelli F, Cirik S, Cossu A, D'Archino R, Djellouli SA, Javel F, Lanfranco E, Mifsud C, Pala D, Panayotidis P, Peirano A, Pergent G, Petrocelli A, Ruitton S, Zuljevic´ A, Ceccherelli G (2005) Invasion of *Caulerpa racemosa* var. *cylindracea* (Caulerpales, Chlorophyta) in the Mediterranean Sea: an assessment of the early stages of spread. Cryptog Algol 26:189-202.

Planas M, Perez-Lorenzo M, Hjelm M, Gram L, Fiksdal IU, Berghc O, Pintado J (2006) Probiotic effect in vivo of Roseobacter strain 27-4 against *Vibrio (Listonella) anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. Aquacult 255:323–333.

Prado S, Romalde JL, Montes J, Barja JL. (2005) Pathogenic bacteria isolated from disease outbreaks in shellfish hatcheries. First description of *Vibrio neptunius* as an oyster pathogen. Dis Aquat Org 67:209-215.

Raguénès G, Christen R, Guezennec J, Pignet P, Barbier G (1997) *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. Int J Syst Bacteriol 47(4):989-95.

Rosowski JR (1992) Specificity of bacterial attachment sites on the filamentous diatom *Navicula confervacea* (Bacillariophyceae). Can J Microbiol 38:676–686.

Ruitton S, Verlaque M, Boudouresque CF (2005) Seasonal changes of the introduced *Caulerpa racemosa* var. *cylindracea* (Caulerpales, Chlorophyta) at the northwest limit of its Mediterranean distribution. Aquat Bot 82:55–70.

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Salaün S, La Barre S, Dos Santos-Goncalvez M, Potin P, Haras D, Bazire A (2012) Influence of Exudates of the Kelp *Laminaria Digitata* on Biofilm Formation of Associated and Exogenous Bacterial Epiphytes. Microb Ecol 64:359-369.

Sambrook J, Russell DW (2001) Molecular Cloning. A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Santavy DL, Colwell RR (1990) Comparison of bacterial communities associated with the Caribbean sclerosponge *Ceratoporella nicholsoni* and ambient seawater. Mar Ecol Prog Ser 67:73–82.

Sieburth JM, Conover JT (1965) *Sargassum* Tannin, an antibiotic which retards fouling. Nature 208:52-53.

Singh RP, Reddy CRK (2014) Seaweed–microbial interactions: key functions of seaweed‐associated bacteria. FEMS microbiol ecol 88(2):213-230.

Sneed JM, Pohnert G (2011) The green alga *Dicytosphaeria ocellata* and its organic extracts alter natural bacterial biofilm communities. Biofouling 27(4):347-356.

Sober E (1983) Parsimony in Systematics: Philosophical Issues. Annu Rev Ecol Evol Syst 14:335–357.

Stabili L, Gravili C, Piraino S, Boero F, Alifano P (2006) *Vibrio harveyi* associated with *Aglaophenia octodonta* (Hydrozoa, Cnidaria). Microb Ecol 52:603–608.

Streftaris N, Zenetos A (2006) Alien Marine Species in the Mediterranean – the 100 'Worst Invasives' and their Impact. Medit Mar Sci 7:87-118.

Talà A, Lenucci M, Gaballo A, Durante M, Tredici SM, Debowles DA, Pizzolante G, Marcuccio C, Carata E, Piro G, Carpita NC, Mita G, Alifano P (2013) *Sphingomonas cynarae* sp. nov., a proteobacterium that produces an unusual type of sphingan. Int J Syst Evol Microbiol 63:72–79.

Thompson FL, Li Y, Gomez-Gil B, Thompson CC, Hoste B, Vandemeulebroecke K, Rupp GS, Pereira A, De Bem MM, Sorgeloos P, Swings J (2003a) *Vibrio neptunius* sp. nov., *Vibrio brasiliensis* sp. nov. and *Vibrio xuii* sp. nov., isolated from the marine aquaculture environment (bivalves, fish, rotifers and shrimps). Int J Syst Evol Microbiol 53:245–252.

Thompson FL, Thompson CC, Li Y, Gomez-Gil B, Vandenberghe J, Hoste B, Swings J (2003b) *Vibrio kanaloae* sp. nov., *Vibrio pomeroyi* sp. nov. and *Vibrio chagasii* sp. nov., from sea water and marine animals. Int J Syst Evol Microbiol 53(3):753-759.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Thomsen MS, Wernberg T, Olden JD, Griffin JN, Silliman BR (2011) A framework to study the context-dependent impacts of marine invasions. J Exp Mar. Biol Ecol 400:322-327.

Tubiash HS, Chanley PE, Leifson E (1965) Bacillary necrosis, a disease of larval and juvenile bivalve mollusks, I. Etiology and epizootiology. J Bacteriol 90:1036–1044.

Tubiash HS, Colwell RR, Sakazaki R (1970) Marine vibrios associated with bacillary necrosis, a disease of larval and juvenile bivalve mollusks. J Bacteriol 103:2721–2723.

Urbanczyk H, Yoshitoshi O, Hayashi T (2013) Taxonomic revision of Harveyi clade bacteria (family Vibrionaceae) based on analysis of whole genome sequences. Int J Syst Evol Microbiol 63(7):2742-2751.

Vairappan C, Anangdan S, Tan K, Matsunaga S (2010) Role of secondary metabolites as defense chemicals against ice-ice disease bacteria in biofouler at carrageenophyte farms. J Appl Phycol 22:305–311.

Verlaque M, Durand C, Huisman JM, Boudouresque CF, Le Parco Y (2003) On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). Eur J Phycol 38:325–339.

Vidgen M, Carson J, Higgins M, Owens L (2006) Changes to the phenotypic profile of *Vibrio harveyi* when infected with the *Vibrio harveyi* myovirus-like (VHML) bacteriophage. J Appl Microbiol 100:481–487.

Vigliotta G, Nutricati E, Carata E, Tredici SM, De Stefano M, Pontieri P, Massardo DR, Prati MV, De Bellis L, Alifano P (2007) *Clonothrix fusca* Roze 1896, a filamentous, sheathed, methanotrophic gammaproteobacterium. Appl Environ Microbiol 73:3556–3565.

Wahl M, Jensen PR, Fenical W (1994) Chemical control of bacterial epibiosis on ascidians. Mar Ecol-Progr Ser 110:45-45.

Wahl M, Shahnaz L, Dobretsov S, Saha M, Symanowski F, David K, Lachnit T, Vasel M, Weinberger F (2010) Ecology of antifouling resistance in the bladder wrack *Fucus vesiculosus*: patterns of microfouling and antimicrobial protection. Mar Ecol-Prog Ser 411:33-48.

Wahl M, Goecke F, Labes A, Dobretsov S, Weinberger F. (2012) The second skin: ecological role of epibiotic biofilms on marine organisms. Front Microbiol 3.

Walls JT, Ritz DA, Blackman MJ (1993) Fouling, surface bacteria and antibacterial agents of four bryozoan species found in Tasmania, Australia. Exp Mar Biol Ecol 169:1–13.

West PA, Colwell RR (1984) Identification and classification of Vibrionaceae: an overview. In: Colwell RR (ed) Vibrios in the Environment. John Wiley, New York, pp 205–363.

Yoshizawa S, Wada M, Kita-Tsukamoto K, Ikemoto E, Yokota A, Kogure K (2009) *Vibrio azureus* sp. nov., a luminous marine bacterium isolated from seawater. Int J Syst Evol Microbiol 59(7):1645-1649.

Yoshizawa S, Wada M, Yokota A, Kogure K (2010) *Vibrio sagamiensis* sp. nov., luminous marine bacteria isolated from sea water. J Gen Appl Microbiol 56(6):499-507.

Yoshizawa S, Tsuruya Y, Fukui Y, Sawabe T, Yokota A, Kogure K, Higgins M, Carson J, Thompson FL (2012) *Vibrio jasicida* sp. nov., a member of the Harveyi clade, isolated from marine animals (packhorse lobster, abalone and Atlantic salmon). Int J Syst Evol Microbiol 62(8):1864-1870.

Zheng L, Han X, Chen H, Lin W, Yan X (2005) Marine bacteria associated with marine macroorganism: the potential antimicrobial resources. Annal Microbiol 55:35-40.

3.7. Supplementary Materials. Chapter III

Table S1
Morphological, cultural and biochemical properties of *Vibrio tubiashii* (CR-I-7, CR-II-17, CR-III-21, CR-IV-32).

Characteristics/tests	*Isolate* (CR-I-7, CR-II-17, CR-III-21, CR-IV-32*)*	*Noguerola and Blanch (2008)*	*Hada et al. (1984)*
Gram reaction	-	-	-
Cell morphology	r	r	r
Luminescence	-	-	-
0/129 sensitivity			
10 µg	+	+	+
Dihydrolase			
Arginine	+	+	+
Decarboxylase			
Lysine	-	-	-
Ornithine	-	-	-
Growth in % NaCl			
0	-	-	-
3	+	d	+
6	-	d	-
10	-	-	-
Oxidase	+	+	+
Catalase	+	nd	+
Urease	-	-	nd
Esculin hydrolysis	-	-	nd
Gelatinase	+	+	+
ONPG	+	+	+
Indole	+	+	nd
Starch	+	nd	+
NO_3	+	+	+
Growth at °C			
4	-	-	-
30	+	d	+
35	+	d	+
40	-	-	-
Acid from			
Sucrose	+	+	+
Glucose	+	nd	+
Carbon sources			
L-Arabinose	-	-	-
N-acetyl-Glucosamine	+	nd	+
Mannose	+	nd	+
Citrate	+	+	nd
Glucose	+	+	nd
Mannitol	nd	nd	nd
Identification	*V. tubiashii*	*V. tubiashii*	*V. tubiashii* ATCC 19109^T

r: rod shaped; d: diverse; nd: no data; +: positive reaction; -: negative reaction.

Table S2
Morphological, cultural and biochemical properties of *Vibrio neptunius* (C-I-2; CR-II-11, CR-III-24, CR-IV-33) and *Vibrio rotiferianus* (CR-I-4, CR-II-10, CR-III-23, CR-IV-28) .

Characteristics/tests	*Isolate* (CR-I-2 CR-II-11, CR-III-24, CR-IV-33*)*	*Thompson et al. (2003)*	*Isolate* (CR-I-4, CR-II-10, CR-III-23, CR-IV-28*)*	*Gomez-Gil et al. (2003)*
Gram reaction	-	-	-	-
Cell morphology	r	r	r	r
Luminescence	-	-	-	-
O/129 sensitivity				
10 µg	+	+	+	+
Dihydrolase				
Arginine	+	+	-	-
Decarboxylase				
Lysine	-	-	+	+
Ornithine	-	-	+	+
Growth in % NaCl				
0	-	-	-	-
3	+	+	+	+
6	+	+	+	+
10	-	-	-	-
Oxidase	+	+	+	+
Catalase	+	+	nd	nd
Urease	-	-	+	+
Esculin hydrolysis			nd	nd
Gelatinase	+	+	+	+
ONPG	-	-	nd	nd
Indole	+	+	+	+
Starch	nd	nd	nd	nd
NO_3	+	+	nd	nd
Growth at °C				
4	-	-	-	-
30	+	+	+	+
35	+	+	+	+
40	-	-	+	+
Acid from				
Sucrose	+	+	+	+
Glucose	+	+	+	+
Carbon sources				
L-Arabinose	-	-	+	+
N-acetyl-Glucosamine	+	+	+	+
Mannose	+	+	+	+
Citrate	+	+	-	-
Glucose	+	+	+	+
Mannitol	-	-	-	-
Identification	*V. neptunius*	*V. neptunius* LMG 20536^T	*V. rotiferianus*	*V. rotiferianus* LMG 21460^T

r: rod shaped; d: diverse; nd: no data; +: positive reaction; -: negative reaction.

Table S3
Morphological, cultural and biochemical properties of *Vibrio jasicida* (CR-I-1, CR-II-14, CR-III-19, CR-IV-32), *Vibrio communis* (CR-I-8, CR-II-14, CR-III-20, CR-IV-25) and *Vibrio owensii* (CR-I-3, CR-II-15, CR-III-19, CR-IV-29) .

Characteristics/tests	*Isolate* (CR-I-1, CR-II-13, CR-III-18, CR-IV-31*)*	*Yoshizawa et al. (2012)*	*Isolate* (CR-I-8, CR-II-14, CR-III-20, CR-IV-25*)*	*Chimetto et al. (2011)*	*Isolate* (CR-I-3, CR-II-15, CR-III-19, CR-IV-29*)*	*Cano-Gomez et al. (2010)*
Gram reaction	-	-	-	-	-	-
Cell morphology	r	r	r	r	r	r
Luminescence	+	+	nd	nd	-	-
O/129 sensitivity						
10 µg	d	d	nd	nd	+	+
Dihydrolase						
Arginine	-	-	-	-	-	-
Decarboxylase						
Lysine	+	+	+	+	+	+
Ornithine	+	+	+	+	+	+
Growth in % NaCl						
0	-	-	-	-	-	-
3	+	+	+	+	+	+
6	+	+	+	+	+	+
10	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	nd	nd
Urease	+	+	-	-	-	-
Esculin hydrolysis	+	+	nd	nd	+	+
Gelatinase	+	+	+	+	+	+
ONPG	+	+	+	+	-	-
Indole	+	+	+	+	+	+
Starch	+	+	+	+	nd	nd
NO_3	+	+	+	+	+	+
Growth at °C						
4	-	-	-	-	-	-
30	+	+	+	+	+	+
35	+	+	+	+	+	+
40	-	-	-	-	-	-
Acid from						
Sucrose	-	-	+	+	+	+
Glucose	+	+	+	+	+	+
Carbon sources						
L-Arabinose	-	-	-	-	-	-
N-acetyl-Glucosamine	nd	nd	+	+	nd	nd
Mannose	+	+	+	+	+	+
Citrate	+	+	-	-	-	-
Glucose	+	+	+	+	+	+
Mannitol	nd	nd	+	+	+	+
Identification	*V. jasicida*	*V. jasicida* TCFB 0772^T	*V. communis*	*V. communis* R-40496^T	*V. owensii*	*V. owensii* DY05^T

r: rod shaped; d: diverse; nd: no data; +: positive reaction; -: negative reaction.

Table S4
Morphological, cultural and biochemical properties of *Vibrio campbellii* (CR-IV-26) and *Vibrio harveyi* (CR-III-22, CR-IV-27)

Characteristics/tests	*Isolate* (CR-IV-26)	*Noguerola and Blanch (2008)*	*Isolate* (CR-III-22, CR-IV-27)	*Noguerola and Blanch (2008)*
Gram reaction	-	-	-	-
Cell morphology	r	r	r	r
Luminescence	-	-	d	d
O/129 sensitivity				
10 µg	d	d	d	d
Dihydrolase				
Arginine	-	-	-	-
Decarboxylase				
Lysine	d	d	+	+
Ornithine	-	-	+	+
Growth in % NaCl				
0	-	-	-	-
3	+	+	+	+
6	d	d	+	+
10	-	-	-	-
Oxidase	+	+	+	+
Catalase	nd	nd		
Urease	-	-	d	d
Esculin hydrolysis	d	d	d	d
Gelatinase	+	+	+	+
ONPG	-	-	d	d
Indole	+	+	+	+
Starch	nd	nd	nd	nd
NO_3	+	+	+	+
Growth at °C				
4	-	-	-	-
30	+	+	+	+
35	+	+	+	+
40	-	-	d	d
Acid from				
Sucrose	-	-	-	-
Glucose	nd	nd	nd	nd
Carbon sources				
L-Arabinose	-	-	+	+
N-acetyl-Glucosamine	nd	nd	nd	nd
Mannose	nd	nd	nd	nd
Citrate	d	d	d	d
Glucose	+	+	+	+
Mannitol	d	d	+	+
Identification	*V. campbelli*	*V. campbellii*	*V. harveyi*	*V. harveyi*

r: rod shaped; d: diverse; nd: no data; +: positive reaction; -: negative reaction.

Table S5
Morphological, cultural and biochemical properties of *Vibrio maritimus* (CR-I-5) and *Vibrio diabolicus* (CR-I-6,CR-II-16)

Characteristics/tests	*Isolate* (CR-I-5, CR-IV-34)	*Chimetto et al. (2011)*	*Isolate* (CR-I-6, CR-II-16)	*Raguenes et al. (1997)*
Gram reaction	-	-	-	-
Cell morphology	r	r	r	r
Luminescence	nd	nd	-	-
O/129 sensitivity				
10 µg	nd	nd	+	+
Dihydrolase				
Arginine	+	+	-	-
Decarboxylase				
Lysine	-	-	+	+
Ornithine	-	-	+	+
Growth in % NaCl				
0	-	-	-	-
3	+	+	+	+
6	+	+	+	+
10	-	-	nd	nd
Oxidase	+	+	+	+
Catalase	+	+	+	+
Urease	-	-	-	-
Esculin hydrolysis	nd	nd	-	-
Gelatinase	+	+	+	+
ONPG	nd	nd	-	-
Indole	+	+	+	+
Starch	nd	nd	+	+
NO_3	+	+	+	+
Growth at °C				
4	-	-	-	-
30	+	+	+	+
35	+	+	+	+
40	-	-	+	+
Acid from				
Sucrose	+	+	+	+
Glucose	+	+	nd	nd
Carbon sources				
L-Arabinose	-	-	-	-
N-acetyl-Glucosamine	+	+	+	+
Mannose	+	+	+	+
Citrate	-	-	+	+
Glucose	+	+	+	+
Mannitol	+	+	+	+
Identification	*V. maritimus*	*V. maritimus* R-40493^T	*V. diabolicus*	*V. diabolicus* HE800^T

r: rod shaped; d: diverse; nd: no data; +: positive reaction; -: negative reaction.

Table S6

Morphological, cultural and biochemical properties of *Vibrio pomeroyi* (CR-II-9) and *Vibrio crassostreae* (CR-II-12).

Characteristics/tests	*Isolate (CR-II-9)*	*Noguerola and Blanch (2008)*	*Thompson et al. (2003)*	*Isolate (CR-II-12)*	*Noguerola and Blanch (2008)*	*Faury et al. (2004)*
Gram reaction	-	-	-	-	-	-
Cell morphology	r	r	r	r	r	r
Luminescence	-	-	-	nd	nd	nd
0/129 sensitivity						
10 µg	+	+	+	+	nd	+
Dihydrolase						
Arginine	+	+	+	+	+	+
Decarboxylase						
Lysine	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-
Growth in % NaCl						
0	-	-	-	-	-	-
3	+	+	+	+	+	+
6	+	+	+	+	+	+
10	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	nd	+	+	nd	+
Urease	-	-	-	-	-	-
Esculin hydrolysis	nd	nd	nd	+	+	+
Gelatinase	+	+	+	+	+	+
ONPG	+	+	+	-	-	-
Indole	+	+	+	+	+	+
Starch	+	nd	+	+	nd	+
NO_3	+	+	+	+	+	+
Growth at °C						
4	+	+	+	+	+	+
30	+	+	+	+	nd	+
35	-	-	-	-	nd	-
40	-	-	-	-	nd	-
Acid from						
Sucrose	-	-	-	+	+	+
Glucose	+	nd	+	nd	nd	nd
Carbon sources						
L-Arabinose	-	-	-	-	-	-
N-acetyl -Glucosamine	+	nd	+	+	nd	+
Mannose	nd	nd	nd	+	nd	+
Citrate	-	-	-	-	-	-
Glucose	+	+	+	+	+	+
Mannitol	+	nd	+	+	nd	+
Identification	*V. pomeroyi*	*V. pomeroyi*	*V. pomeroyi* LMG 20537^T	*V. crassostreae*	*V. crassostreae*	*V. crassostreae* CAIM 1405^T

r: rod shaped; d: diverse; nd: no data; +: positive reaction; -: negative reaction.

Table S7
Morphological, cultural and biochemical properties of *Vibrio aestivus* (CR-IV-30).

Characteristics/tests	*Isolate (CR-IV-30)*	*Lucena et al. (2012)*
Gram reaction	-	-
Cell morphology	r	r
Luminescence	nd	nd
O/129 sensitivity		
10 µg	nd	nd
Dihydrolase		
Arginine	-	-
Decarboxylase		
Lysine	-	-
Ornithine	-	-
Growth in % NaCl		
0	-	-
3	+	+
6	+	+
10	-	-
Oxidase	+	+
Catalase	+	+
Urease	nd	nd
Esculin hydrolysis	nd	nd
Gelatinase	-	-
ONPG	+	+
Indole	-	-
Starch	-	-
NO_3	+	+
Growth at °C		
4	-	-
30	+	+
35	+	+
40	-	-
Acid from		
Sucrose	-	-
Glucose	+	+
Carbon sources		
L-Arabinose	-	-
N-acetyl-Glucosamine	+	+
Mannose	-	-
Citrate	+	+
Glucose	+	+
Mannitol	-	-
Identification	*V. aestivus*	*V. aestivus* CECT 7558^T

r: rod shaped; d: diverse; nd: no data; +: positive reaction; -: negative reaction.

4. Functional and molecular diversity of the microbial community living on the invasive alga *Caulerpa cylindracea* surface

4.1. Abstract

The green alga *Caulerpa cylindracea* (Sonder), is an invasive species causing environmental concern in the Mediterranean countries, where it represents a serious threat for native assemblages. Understanding the ecological processes potentially involved in the successful spreading of the algae is critical to provide further insights in patterns of invasions. We combined cultural and molecular approaches to analyze the bacterial community profiles associated to the surface of the Mediterranean invasive alga *C. cylindracea* collected in different sites across the central Mediterranean area, far apart hundreds of kilometers each other. The hypothesis is that this bacterial community could provide specific functional properties to the algae influencing its ability to colonize new areas. Hence, we used the Biolog system - Ecoplates ™ (Biolog, Hayward, CA, USA), in conjunction with polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) to acquire information on the functional and phylogenetic diversity of the algal associated bacterial assemblages. Culturable heterotrophic bacteria at 22 °C showed a mean value of 2.4 x 10^5 CFU mL^{-1} at Torre Guaceto (Italy), 2.2 x 10^5 CFU mL^{-1} at Otranto (Italy), 1.4 x 10^5 CFU mL^{-1} at Porto Cesareo (Italy), 5 x 10^6 CFU mL^{-1} at Bay of Kotor (Montenegro) and 5.8 x 10^6 CFU mL^{-1} at Othonoi, Diapontie Island (Greece). The analysis of the metabolic profiles revealed that the greatest metabolic diversity was found in the *C. cylindracea* Montenegro and Greece inocula, which contained 20 and 19 degraded carbon sources respectively. Great diversity was also found in the *C. cylindracea* inocula from Otranto, Torre Guaceto and Porto Cesareo, each of them including 18 eco-substrates consumed in a week. A high similarity in the bacterial profiles among the study sites was observed (always higher than 76%). The Ez-taxon analysis of the DNA sequences demonstrated that there is a distinct group of bacteria (i.e. the genera *Shewanella*, *Marinobacter*, *Vibrio*, *Granulosicoccus* and an unknown strain belonging to Rhodobacteraceae family) consistently present on *C. cylindracea*, irrespective of its

geographical origin. *Caulerpa cylindracea* hosts a specialized bacterial community with a functional diversity and a metabolic versatility potentially playing a crucial role in patterns of algal invasions.

4.2. Introduction

In the marine environment, bacteria regulate rates of organic matter mineralization, nutrient cycling, and energy transfer through their metabolism in two major ways: by production of new bacterial biomass (secondary production) and by re-mineralization of organic carbon and nutrients. These ecosystem processes are commonly limited by the quantity and quality of resources. Natural dissolved organic matter (DOM) sources are heterogeneous mixtures of compounds, and bacteria can utilize several molecules simultaneously (Bott and Kaplan, 1985; Cavallo et al., 1999). In particular the polysaccharides, proteins and lipids produced as a result of carbon fixation together form the core of DOM and POM (particulate organic matter) pools. All bacterial metabolic processes begin with the interaction between the microbial cell wall or membrane and the external environment. Extracellular enzymes (EE) are necessary for bacterial feeding because most utilizable marine DOM and all POM is too large to be taken into bacterial cells without first being hydrolyzed into lower molecular weight compounds (Cho and Azam, 1988; Smith et al., 1992; Amon and Benner, 1996) and only relatively small molecules (less than around 600 Da) (Weiss et al., 1991) can be transported across the bacterial membrane. Microbial EE may control organic polymer degradation and utilization (Hoppe, 1991; Meyer-Reil, 1991) and be coupled with bacterial substrate uptake and growth (Ammerman and Azam, 1985; Hoppe et al., 1993).

Released enzymes with diverse specificities include proteases, chitinases, cellulases, amylases, phosphatases, and others (Inagaki, 1991; Dick et al., 1992; Lemke et al., 1995; Uchida, 1995; Svitil et al., 1997). The metabolic diversity of bacteria is perhaps as remarkable as their taxonomic and evolutionary diversity. In the case of high-molecular-weight DOM, diverse group of bacteria may consume it since specific extracellular enzymes are required for the hydrolysis of biopolymers, a component of high-molecular weight DOM. Van Hannen et al. (1999) showed that the quality of DOM influenced the bacterial community structure. Moreover, even small additions of organic substrates may trigger a shift in the composition of the microbial community and an accompanying change in the relative abundance of specific hydrolytic ectoenzymes (Pinhassi et al., 1999). Thus, microbial communities may respond to a varying supply of substrates either by physiological adaptation or by changes in the community composition (Cavallo et al., 1999).

In the present study, we combined cultural and molecular approaches to analyze and

compare the bacterial community profiles associated to the surface of the Mediterranean invasive alga *Caulerpa cylindracea* collected in different sites across the central Mediterranean Sea, far apart hundreds of kilometers each other. *Caulerpa cylindracea* (Sonder) (Belton et al., 2014), previously known as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman and Boudouresque (Verlaque et al., 2003), is a non-indigenous invasive algal species introduced in the Mediterranean Sea from the Red Sea and observed for the first time in Libya in 1990 (Klein and Verlaque, 2008). From then on, it has colonized all available habitats and substrates leading to profound structural and functional alterations of indigenous benthic assemblages and fish metabolism (Holmer et al., 2009; Cebrian et al., 2012; Felline et al., 2012). Here, the bacterial communities associated to the surface of the marine green macroalga *C. cylindracea* has been studied to provide specific insights on a component that might have a role in driving the trajectory of invasion of this algae across the basin. More specifically, the hypothesis is that this bacterial community could provide specific functional properties related to the utilization of the different carbon substrates potentially influencing its ability to colonize new areas. Previous attempts (Aires et al., 2013) revealed that endobacterial communities can be an effective tracer of the origin of invasion and support their potential role in their eukaryotic host's adaptation to new environments. In this study, the Biolog EcoPlate system (Biolog, Hayward, CA, USA), in conjunction with polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) was used to describe the functional and phylogenetic diversity of the bacterial assemblages present on *C. cylindracea* surface. Metabolic assays mainly based on carbohydrates degradation are still effectively used to identify environmental bacteria besides molecular methods. Among these methods the Biolog EcoPlates microtiter plates which offers a standardized rapid method for determining bacterial oxidation of 31 ecologically relevant carbon substrates with a redox-sensitive tetrazolium indicator of microbial respiration (Truu et al., 2009). Measurements of substrate use enables qualifying microbial metabolic capabilities and hence functional diversity of a microbial community. The community-level BIOLOG assay has become popular because of its rapidity and simplicity. In this scenario, herein we combined both procedures to improve our understanding of the microbial community diversity associated to *C. cylindracea* surface.

4.3. Material and Methods

Sampling

Caulerpa cylindracea was collected during the bloom in September by SCUBA divers in the Bay of Kotor, Montenegro (42°29'06.6"N, 18°41'28.6"E); Othonoi (Diapontine Islands) Greece (39° 50.257'N, 19° 24.037'E); the Marine Protected Area of Torre Guaceto (Brindisi), Italy (40°42'59.25"N, 17°48'5.12"E), Otranto (Lecce), Italy (40° 9'5.94"N, 18°29'27.16"E) and the Marine Protected Area of Porto Cesareo (Lecce) Italy (N 40°12.772', E017°48.218') at shallow water (5-10 m depth) on a rocky substrate (Fig. 4.1). About 300 g of *C. cylindracea* were collected in triplicate and transferred to the laboratory under controlled temperature and processed for culturable heterotrophic bacteria enumeration within 4 h from sampling.

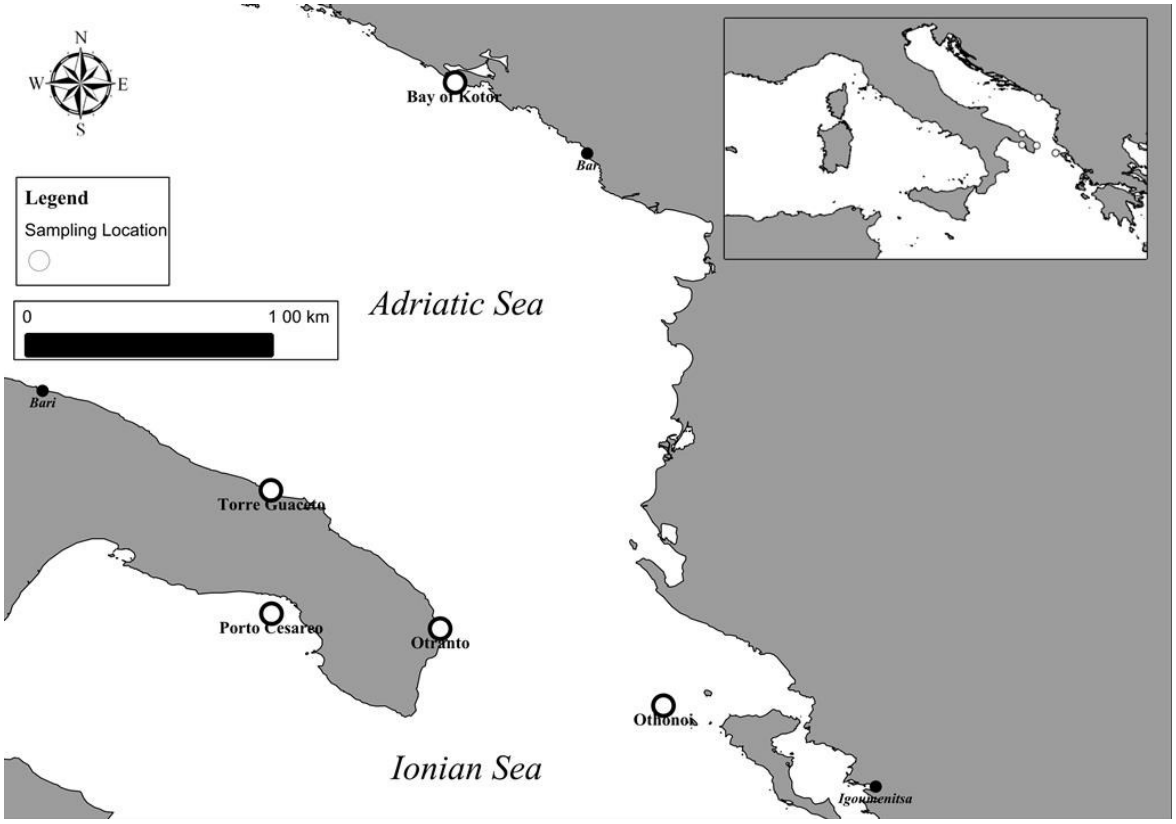


Figure 4.1 Map of the five sampling sites in the Mediterranean Sea.

Bacteria enumeration

In the laboratory, the seaweed was washed several times in sterile seawater (0.2 μm pore filtered) to eliminate the bacteria settled on the surfaces, then suspended in sterile seawater and sonicated three times (Branson Sonifier 2200, 60 W, 47 kHz for 1 min in an ice bath) to optimize surface bacteria detachment. The sonication was interrupted for 30 s every minute, during which time the samples were shaken manually. To enumerate surface bacteria 1 or 5 mL of the sonicated sample and appropriate decimal dilutions were plated onto Marine Agar 2216 and after incubation for 2 days at 22 °C the culturable bacteria were counted according to the colony forming units (CFU) method.

Total bacterial counts were performed using a Zeiss Standard Axioplan microscope equipped with a halogen lA (Hg 100) light. Duplicate slides were prepared from each sample by filtering 1ml of seawater onto a Millipore filter (0.2 mm pore), using DAPI (4,6-diamidino-2 phenyl-indole) as fluorochrome (Porter and Feig, 1980). AG 365 excitation filter, an FT 395 chromatic beam splitter and an LP 420 barrier filter were used. At least 40 microscopic fields were counted for each preparation at 1000 magnification.

Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP), DNA sequencing and phylogenetic trees

Bacteria were collected from *C. cylindracea* (about 1 g) surface as described above, and high molecular weight genomic DNA was extracted as described (Vigliotta et al., 2005). After the extraction, the DNA was amplified using the bacteria-specific primers Com1-F (5'-CAGCAGCCGCGGTAATAC-3') and Com2-R (5'-CCGTCAATTCCTTTGAGTTT-3') targeting 16S rRNA encoding genes (Lane et al., 1985). These primers were designed to amplify 409 bp long DNA fragments (from nucleotide 519 to nucleotide 927 in the *Escherichia coli* 16S rRNA gene) that could be resolved by SSCP as described (Di Giacomo et al., 2007; Stabili et al., 2008; Vigliotta et al., 2007). To this purpose, PCR products were purified by High Pure PCR product Purification Kit (Boehringer Mannheim), denatured and resolved on 10% polyacrylamide gel (acrylamide/N,N-methylenebisacrylamide 49:1) in 0.8 X TBE (72 mM Tris-borate, 1.6 mM EDTA) containing 5% glycerol. Bands identified after silver staining were excised with razorblades and single strand DNAs were eluted from the gel by using the Qiaex II DNA purification kit (Qiagen). DNA similarity searches were carried out using Ez-Taxon-e database (http:// (http://eztaxon-e.ezbiocloud.net, Kim et al., 2012). Sequence alignments

were performed with ClustalW at EBI (http://www.ebi.ac.uk/). Phylogenetic analyses were conducted using the SeaView 4 program (Galtier et al., 1996; Gouy et al., 2010), according to the neighbour-joining (NJ) (Saitou and Nei, 1987), maximum-parsimony (MP) (Sober, 1983), and maximum-likelihood (ML) (Felsenstein, 1981) methods and Kimura's two-parameter algorithm (Kimura, 1980). Bootstrap analysis (Salemi and Vandamme, 2003) was used to estimate the reliability of phylogenetic reconstructions (1,000 replicates).
The 16S rDNA nucleotide sequences were deposited at GenBank with the following accession numbers: Bacteroidetes (KJ660319), *Shewanella* sp. (KJ660320), *Marinobacter* sp. (KJ660321), *Vibrio* sp. (KJ660322), *Granulosicoccus* sp. (KJ660323), Rhodobacteraceae (KJ660324).

BIOLOG ECO plate inoculation and incubation

BIOLOG ECO plate (BIOLOG Inc., Hayward, Calif.) is a system made by a set of 31 substrates and one blank well in triplicate. In this work, three BIOLOG ECO plates were utilized for each algal sonicate, obtaining nine replicates. In each well a volume of 150 μL were inoculated and the BIOLOG ECO plates were incubated at 30 °C for 1 week. The optical density (OD) values were measured at a wavelength of 590 nm with a plate reader and the increase in optical density was recorded in each well, obtained by subtracting the optical density values at the beginning of incubation from the optical density at the end of incubation.

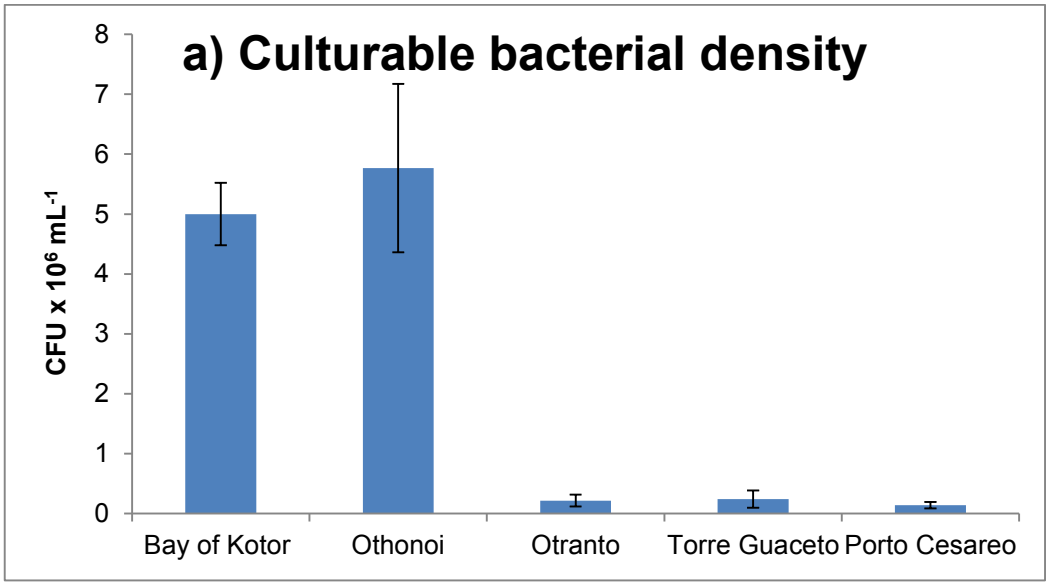
Statistical analysis

The similarity percentages procedure (SIMPER; Clarke, 1993) was used to identify the percentage contribution of similarity of each substrate within each site and among sites. The analysis was done with Bray–Curtis similarity measures (SBC) on square root-transformed data (Primer; Clarke, 1993; Clarke and Warwick, 1994). This transformation comprised all substrates within the same range of abundance, preventing the most abundant optical densities from leading the analyses. The analyses were performed using the computer program PRIMER v. 6 (Clarke and Gorley, 2006).

4.4. Results

Bacteria enumeration

Data concerning bacterial concentrations on *C. cylindracea* surface are reported as mean values in Fig. 4.2a. Culturable heterotrophic bacteria at 22 °C showed a mean value of 2.4 x 10^5 CFU mL^{-1} at Torre Guaceto, 2.2 x 10^5 CFU mL^{-1} at Otranto, 1.4 x 10^5 CFU mL^{-1} at Porto Cesareo, 5 x 10^6 CFU mL^{-1} at Bay of Kotor and 5.8 x 10^6 CFU mL^{-1} at Othonoi. The trends of bacterial abundance in the five sampling locations are shown in Fig. 4.2b. The lowest bacterial concentration is reported in Otranto, the highest one is found in Bay of Kotor.



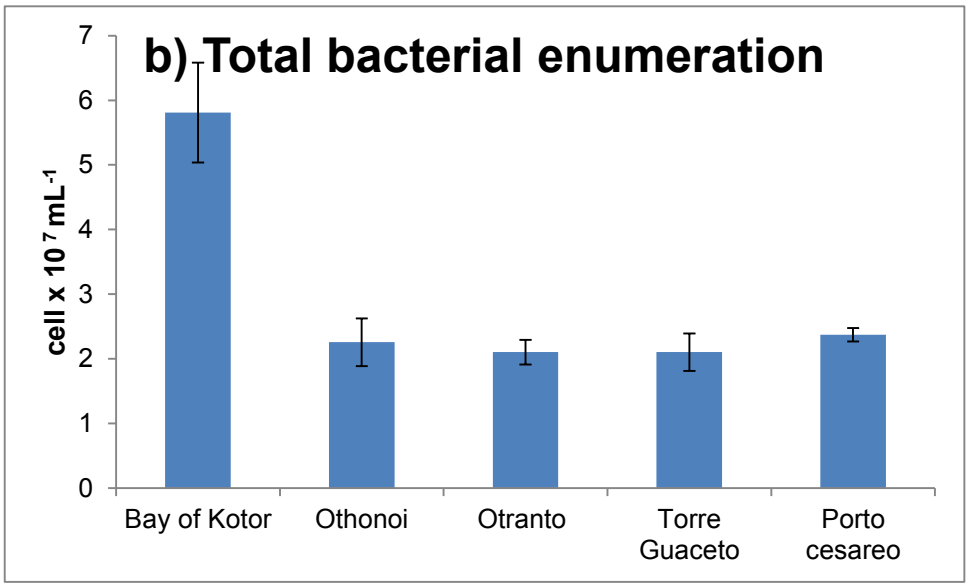


Figure 4.2. Mean abundance and relative standard deviation of a) cultural heterotrophic bacterial densities and b) total bacteria numeration on *C. cylindracea* collected in the five sampling locations. Data are reported as mean values ±S.E.

Bacterial metabolic profiles

The metabolic abilities of the bacterial communities on *C. cylindracea* collected in the different sites were investigated using BIOLOG EcoPlates. The analysis of the physiological profiles of the bacterial communities from the algal surfaces shows that the five algal communities had similar patterns of sole carbon source utilization (Table 4.1).

Table 4.1. Results from BIOLOG ECO plate indicating the utilization of the 31 substrates by the bacterial community on *C. cylindracea* surface collected in the five sampling sites.

	Carbon Sources	Kotor	Othonoi	Otranto	Porto Cesareo	Torre Guaceto
Amino acids	L-Arginine	-	-	+	+	+
	L-Asparagine	+	+	+	+	+
	L-Phenylalanine	+	+	+	+	+
	L-Serine	+	+	+	+	+
	L-Threonine	-	-	-	-	-
	Glycyl-L-Glutamic Acid	+	-	-	-	-
	Phenylethyl-amine	+	+	+	+	+
	Putrescine	+	+	+	+	+
Carbohydrates	β-Methyl-D-Glucoside	+	+	+	+	+
	D-Xylose	+	+	+	+	+
	i-Erythritol	-	-	-	-	-
	D-Mannitol	+	+	-	-	-
	N-Acetyl-D-Glucosamine	-	-	-	-	-
	D-Cellobiose	+	+	+	+	+
	Glucose-1-Phosphate	+	+	+	+	+
	α-D-Lactose	+	+	+	+	+
	D,L-α-Glycerol Phosphate	+	+	+	+	+
Carboxylic Acid	D-Galactonic Acid γ-Lactone	-	+	+	+	+
	Pyruvic Acid Methyl Ester	+	+	+	+	+
	D-Galacturonic Acid	+	+	+	+	+
	2-Hydroxy Benzoic Acid	+	+	-	-	-
	4-Hydroxy Benzoic Acid	+	+	+	+	+
	γ-Hydroxybutyric Acid	-	-	-	-	-
	D-Glucosaminic Acid	-	-	-	-	-
	Itaconic Acid	+	-	-	-	-
	α-Ketobutyric Acid	-	-	-	-	-
	D-Malic Acid	-	-	-	-	-
Polymers	Tween 40	+	+	+	+	+
	Tween 80	+	+	+	+	+
	α-Cyclodextrin	-	-	-	-	-
	Glycogen	-	-	-	-	-

In particular, the greatest metabolic diversity was found in the *C. cylindracea* Montenegro and Grecia inocula, which contained 20 and 19 degraded carbon sources respectively. Great diversity was also found in the inocula of *C. cylindracea* sampled in Otranto, Torre Guaceto and Porto Cesareo, each of which contained 18 eco-substrates consumed in a week. Although some little differences in carbon sources degradation were recorded, in all the examined sites the common metabolized substrates were: D-cellobiose, glucose-1-phosphate, α-D-lactose, D,L-α-glycerol phosphate, β-methyl-D-glucoside, and D-xylose among carbohydrates (Fig. 4.3a); L-asparagine, L-phenylalanine and L-serine among amino acids (Fig. 4.3b); D-galacturonic acid and pyruvic acid methyl ester among carboxylic acids (Fig. 4.3c); Tween 40 and Tween 80 among polymers (Fig. 4.3d); phenylethylamine and putrescine among amines (Fig. 3b), and 4-hydroxy benzoic acid among phenols (Fig. 4.3b). In all the examined sites, carbohydrates were the most preferred substrates over amino acids, polymers, carboxylic acids and esters.

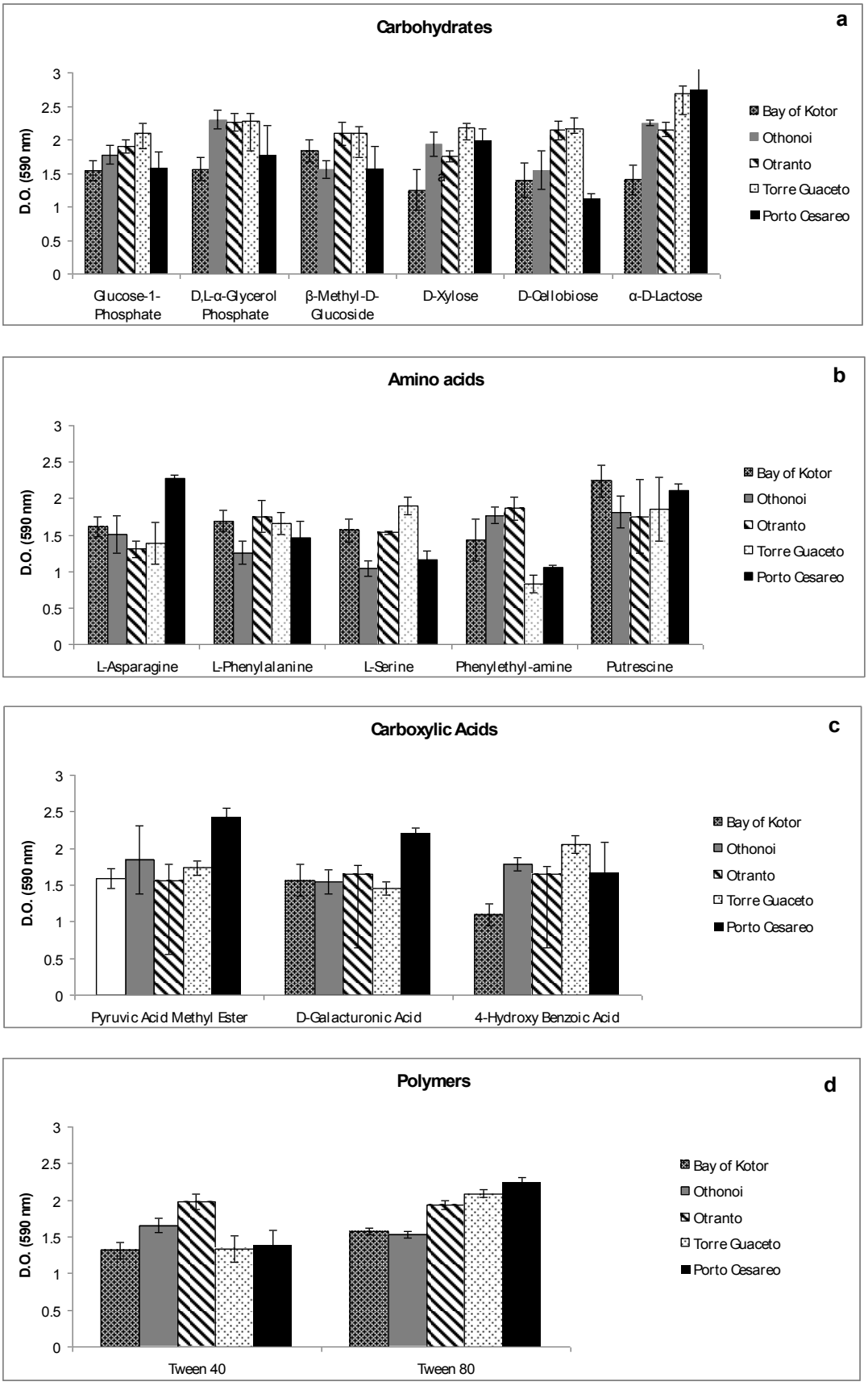


Figure 4.3. Carbohydrates(a), amino acids (b), carboxylic acids (c) and polymers (d) utilization by the bacterial community on *C. cylindracea* surface collected in the five sampling sites. Data are reported as mean values ±S.E.

Statistical analysis

The SIMPER analysis revealed a high similarity among the study sites: in all cases the similarity is higher than 76%. The highest percentages of Bray–Curtis similarity are found in the Italian sites going from a similarity of 90.87% between Otranto and Porto Cesareo to 93.78% between Otranto and Torre Guaceto. The lowest percentages of Bray-Curtis similarity are observed between the Bay of Kotor (Montenegro) and any other site (Table 4.2).

Table 4.2. The SIMPER analysis shows the percentage contribution of Bray–Curtis similarity measures (SBC) of each substrate within each site and among sites. The analysis was performed on square root-transformed data.

	Othonoi	Bay of Kotor	Otranto	Porto Cesareo	Torre Guaceto
Othonoi	94.23				
Bay of Kotor	84.13	93.25			
Otranto	86.82	78.14	95.69		
Porto Cesareo	84.9	76.73	90.87	94.36	
Torre Guaceto	85.52	77.15	93.78	91.37	95.38

PCR-SSCP, DNA sequencing and phylogenetic trees

The findings of Ecoplate Biolog were supported by DNA-based approaches (Fig. 4.4). To this purpose, the epibiotic bacterial communities of *C. cylindracea* sampled from the five different sites of Mediterranean area were analyzed by PCR–SSCP using bacteria-specific primers Com1-F and Com2-R. These primers target a 409-bp-long (in *E. coli*) central region of prokaryotic 16S rRNA gene and allow high taxonomic resolution. The SSCP profile of the amplified 16S rRNA gene pool revealed a relatively low degree of complexity of the microbial communities living on *C. cylindracea* surface with a predominance of a few common bands among samples examined (Fig. 4.4). In particular bands **a**, **b**, **c**, **e** and **m** were common to all the samples consistent with the high metabolic similarity between the different samples shown by Biolog system analysis. Moreover band **h** was common to Othonoi, Otranto, Porto Cesareo and Torre Guaceto.

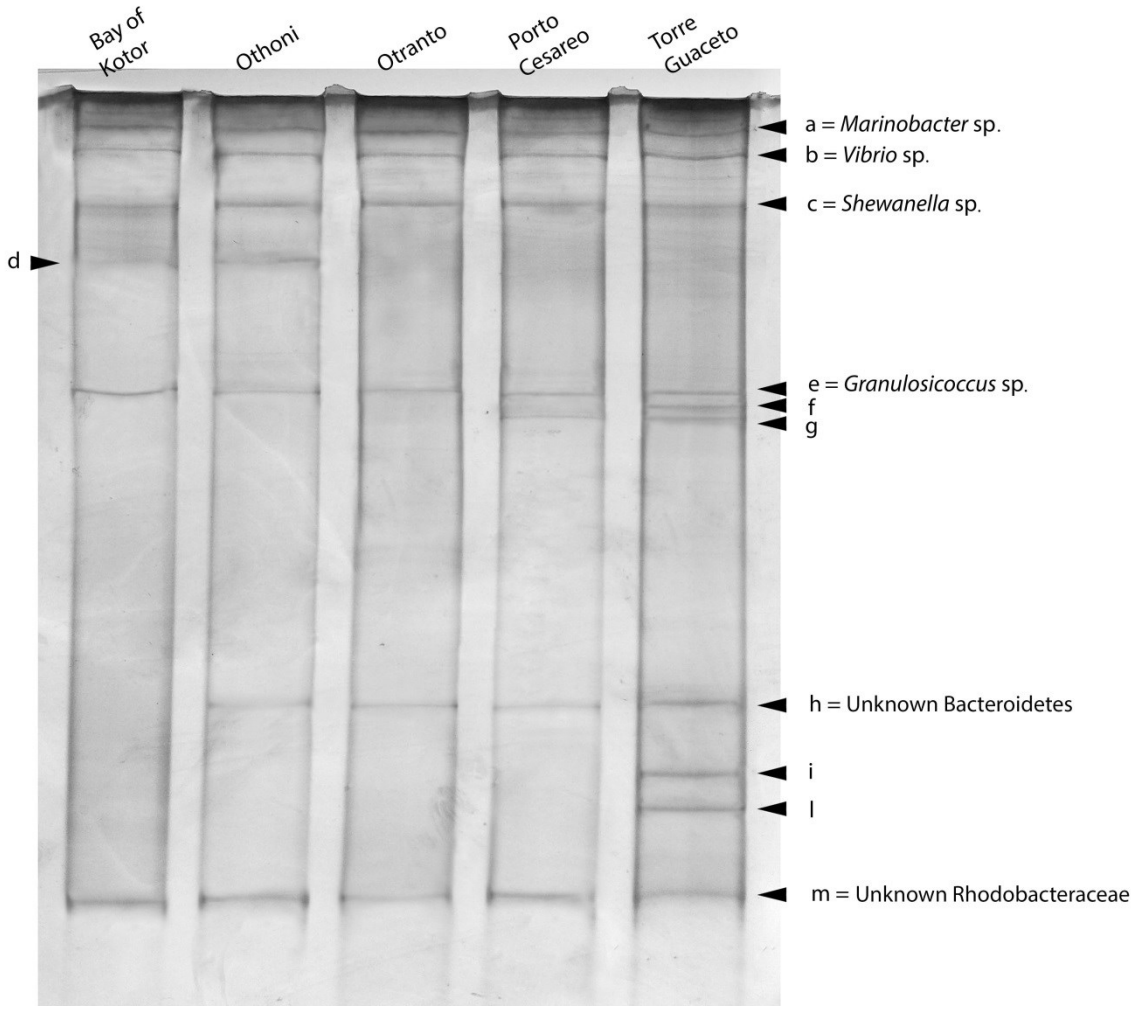


Figure 4.4. PCR–SSCP analysis of the bacterial community on *C. cylindracea* surface. Five distinct batches of *C. cylindracea* sampled in the Bay of Kotor, Othoni, Otranto, Porto Cesareo and Torre Guaceto were analyzed for the presence of epibiotic bacteria by PCR–SSCP using 16S rRNA gene-specific primers. Arrowheads mark the positions of specific DNA bands (a, b, c, d, e, f, g, h, i, l, m).

All the above mentioned bands were excised from the gel, eluted, and subjected to DNA sequencing. The Ez-Taxon analysis of the DNA sequences demonstrated that the bacteria common to all five *C. cylindracea* samples belonged to the genera *Marinobacter, Granulosicoccus,* and *Vibrio.* Most closely-related species were, respectively, *Marinobacter xestospongiae* (99.51 % identity), (% identity), *Granolosicoccus coccoides* (91.57 % identity), *Vibrio sagamiensis* and *Vibrio rotiferianus* (99.71% identity) both belonging to the *V. harveyi* clade as also shown by phylogenetic tree analysis (Fig. 4.5). As regards *Shewanella* the most closely-related species belonged to a robust cluster that enclosed the following species *Shewanella waksmanii*, *Shewanella marisflavi*, *Shewanella pealeana*, *Shewanella piezotolerans*, *Shewanella halifaxensis, Shewanella*

violacea, *Shewanella kaireitica*, *Shewanella schlegeliana*, *Shewanella marinintestina*, *Shewanella sairae* and *Shewanella psychrophila* (98.75 % identity) (Fig. 4.5c.). By contrast band **h**, was assigned to an unknown species of Bacteroidetes phylum (87.40 % identity with *Lewinella marina*) (Fig. 4.5e), and band **m** was assigned to a microorganism belonging to Rhodobacteraceae family (96.11% identity with *Wenxinia marina*) (Fig. 4.5f).

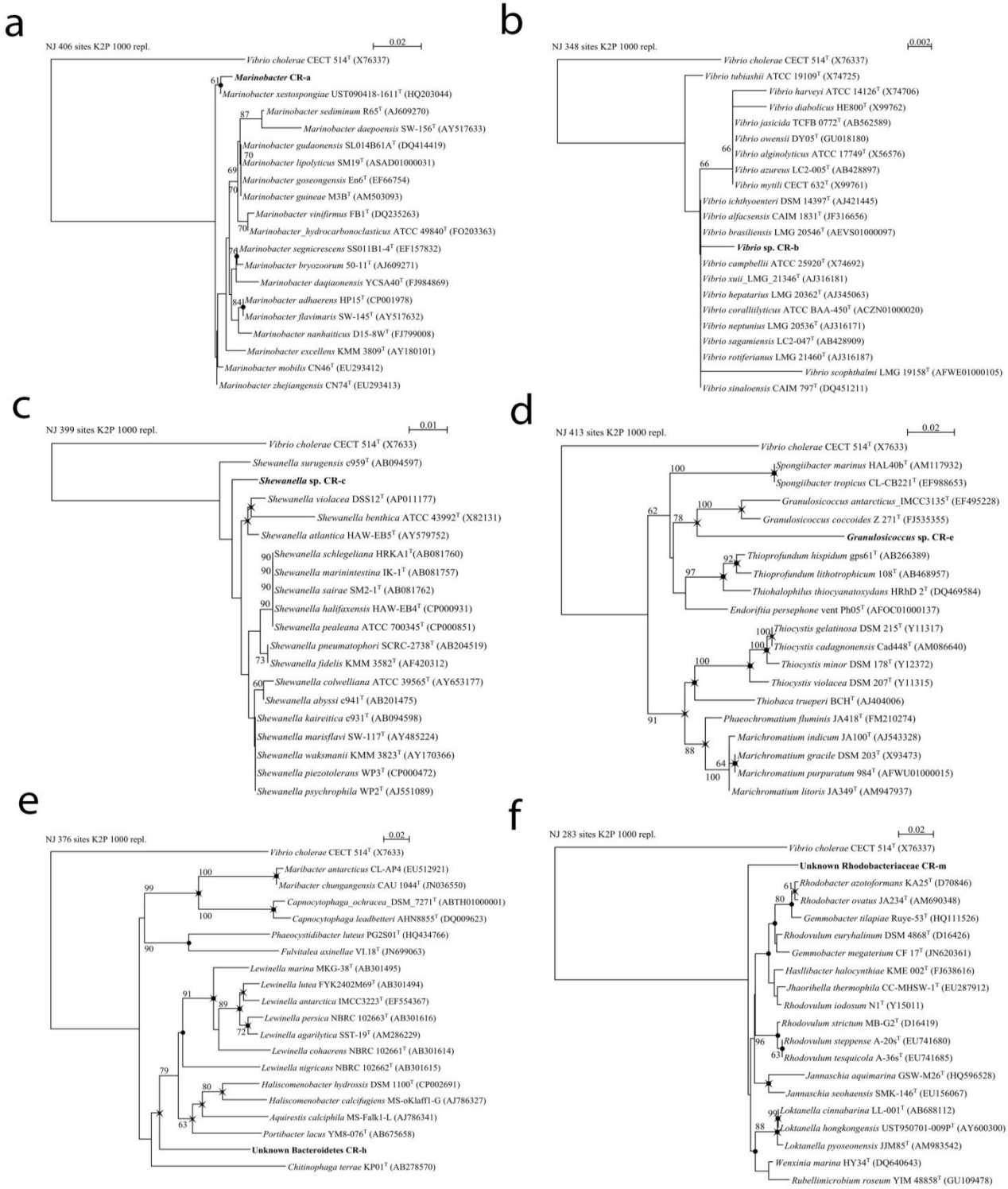


Figure 4.5. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing positions of *Marinobacter* (a) *Vibrio,* (b) *Shewanella*, (c) *Granulosicoccus* (d) genera; Bacteroidetes phylum (e) and Rhodobacteraceae family (f) from *C. cylindracea* surface (in bold) with respect to closely-related species. Bootstrap values (expressed as percentages of 1,000 replicates) of >60 % are shown at branch point. Filled circles and "X" indicate that corresponding nodes were also recovered in phylogenetic trees constructed with maximum parsimony and maximum-likelihood algorithms respectively. *Vibrio cholerae* CECT 514T was used as outgroup.

4.5. Discussion

Here, a combination of different approaches was used to assess the structural and functional diversity of the bacterial community associated to *C. cylindracea* surface.

The use of different approaches, the community-level BIOLOG assay and the DNA-base approaches, to assess the bacterial profiles presents a number of advantages. The Biolog system is an pratical method of differentiating bacterial community. However, this approach is still debatable and the interpretation of the metabolic profiles obtained requires care. To overcome these problems and corroborate our results here we proposed the combination of the bacteria community-level BIOLOG assay with PCR-SSCP and DNA sequencing. The abundance of culturable bacteria on the surface of *C. cylindracea* during the bloom varies from 1.4 x 10^5 CFU mL^{-1}at Porto Cesareo to 5 x 10^7 CFU mL^{-1} at Montenegro. These results are consistent with those obtained in other studies showing that seaweeds and seagrasses have dense populations of bacteria (up to 10^6 per cm^2) on their surfaces, although this varies considerably with species, geographic location and climatic conditions (Provasoli and Pintner, 1980; Shiba, 1992; Chisholm et al., 1995; Meusnier et al., 2001). In particular, the genus *Caulerpa* has long been known to harbor endosymbiotic and epiphytic bacteria, which may be associated with various metabolic functions including nitrogen fixation and/or the production of various toxic compounds. Plant-associated bacteria usually exchange signals with their hosts and possess the ability to colonize plant surfaces and tissues, to metabolize plant-derived carbon sources and to synthesize plant hormones (Preston et al., 1998).

The results of BIOLOG assay suggests that the bacterial communities associated with *C. cylindracea* surface have similar metabolic profiles in all five sites examined, and have a great potential to utilize carbon sources. Due to this functional diversity and metabolic versatility these epibiotc bacteria might perform several substrate transformations with presumably profound implications on the algal spreading. In particular we observed that D-Cellobiose, Glucose-1-Phosphate, α-D-Lactose, D,L-α-Glycerol Phosphate, β-Methyl-D-Glucoside, and D-xylose are among the utilized carbohydrates. In estuarine and marine surface waters carbohydrates are considered the most labile fractions of bulk organic matter and may play key roles in the geochemical cycles as reported by several studies (Burdige and Zheng, 1998; Murrell and Hollibaugh, 2000; Benner, 2002). Some carbohydrates might be algal-derived carbohydrates and associations between algae and

bacteria have been elucidated by the benefits provided to the bacteria, such as support of bacterial growth by dissolved organic carbon released by algal cells (Rier and Stevenson, 2002; Rao et al., 2006). The utilization of the six carbohydrates by *C. cylindracea* associated bacteria might support this hypothesis since xylose and glucose, for example, have been reported as main components of polysaccharides from *C. cylindracea* (Chattopadhyay et al., 2007). Morever D-xylose and (4)-hydroxy benzoic acid have been reported as algal constituents, as well as other compounds extensively metabolized by the here investigated bacterial communities, such as L-phenylalanine, L-asparagine, L serine which are common components of living organisms (Lachnit et al., 2010; Rajasulochana et al., 2013). Among polymers Tween 40 and Tween 80 were utilized by the bacteria associated to *C. cylindracea*. High utilization of Tween 40 and Tween 80 has also been described for other microbial communities (Sala et al., 2005; Sala et al., 2008). Polyols, such as Tween 40 and Tween 80, are suggested to be accumulated by algae and fungi in order to grow also at low temperatures (Robinson, 2001; Sala et al., 2008). D-galacturonic acid and pyruvic acid methyl ester were among the utilized carboxylic acids. Carboxylic acids are an important carbon source for bacterioplankton in the Mediterranean Sea and other aquatic environments (Obernosterer et al., 1999; Pullin et al., 2004; Sala et al., 2006) and are considered part of the labile pool of organic matter. Pyruvic acid methyl ester has been recorded in *Caulerpa taxifolia* (Mancini et al., 1998) and D-galacturonic acid as a component of other green algae (Khotimchenko, 1995). Phenylethylamine and putrescine were among the utilized amines.

Results of the present study found few metabolic differences when comparing bacterial assemblages on *C. cylindracea* surface from the different sites demonstrating that the alga surface harbours a specialized bacterial community. By PCR–SSCP analysis we found molecular traces of the presence of gamma-Proteobacteria belonging to the genera *Shewanella*, *Marinobacter*, *Vibrio* and *Granulosicoccus*, along with those of an unknown species belonging to the Rhodobacteraceae family on the surface of *C. cylindracea* in all five investigated sites (Fig. 4.4 and 4.5). Moreover, traces of an unknown species of the Bacteroidetes phylum were found in four out of five samples. Although in most of cases the lengths of the amplified 16S rRNA sequences and their homologies in the databank were not sufficient to assign the putative species name to each PCR–SSCP bands, the sequence of band **a** (Fig. 4.4 and 4.5) exhibited considerable identity (99.51%) with that of *Marinobacter xestospongiae*. This microorganism, which belongs to a genus of oil-degrading bacteria (Yakimov et al., 2007), was recently isolated from the marine sponge

Xestospongia testudinaria collected from the Red Sea (Lee et al., 2012). In this context, it is also interestingly to stress the ability of *Granulosiccoccus coccoides*, strictly related to the bacterial strain in band **e (**Fig. 4.4 and 4.5**)**, to degrade Tweens 2, 4 and 80, consistently with the results of BIOLOG assay (Fig. 4.3 and Table 4.1).

Aries et al. (2013) found, through tag-Pyrosequencing, that Flavobacteriales, Sphingobacteriales and Rhodobacterales were the most prevalent orders of epiphytic bacteria associated to *C. cylindracea* samples from Mediterranean Sea and Australia. However, in that study bacterial metabolism was only inferred from 16S characterization. By contrast, in the present study, we acquired information on the functional and phylogenetic diversity of the algal associated bacterial assemblages using the Biolog system - Ecoplates in conjunction with PCR-SSCP.

The seaweed surface may play an important role in driving the development of specific bacterial communities, since provides a suitable substrate for the settlement of microorganisms and also secretes various organic substances providing nutrients for multiplication of bacteria or discourage the grown of microbiota (Sneed and Pohnert, 2011; Singh and Reddy, 2014). The algal morphology and micro-topography of surface may also influence associated bacterial biofilm, so each seaweed represents a unique micro-ecosystem (Wahl et al., 2010). Moreover, bacteria growing on the surfaces of marine algae live in a highly competitive environment where space and access to nutrients are limited. Once established, particular microbes may themselves influence colonization by other fouling organisms. Thus, microbial communities living on the seaweed surface are highly complex, dynamic systems and consist of a consortium of microorganisms including bacteria, fungi, diatoms, protozoa, spores and larvae of marine invertebrates (Goecke et al., 2010; Lachnit et al., 2011; Singh and Reddy, 2014). Previous studies on the other invasive algae *C. taxifolia* (Meusnier et al., 2001) and *C. cylindracea* (Aires et al., 2013) have shown that an important bacterial community is associated with their entire thalli. These observations lead to hypothesize that the algal host obtain clear benefits from the microbial association relying on secondary metabolites produced by surface-associated bacteria as their defense against fouling. Thus, the competitive potential of invasive species may not be entirely determined by their intrinsic capacities, but may be at least partly shaped by associated microbes as already suggested by Byers et al. (2010). Our understanding of the mechanisms underlying the establishment and spread of introduced species may therefore require a serious appraisal of the potential co-introduction and influence of bacterial communities on the success of non-indigenous

species as already underlined by Areis et al. (2013).

At moment in the association we documented in this study, it is not known whether the seaweed-associated bacteria are strictly host-specific. However, the diversity found in this component can determine their adaptation to new environments and their capacity to outcompete native organisms. On account of our results we might hypothesize that the interaction between *C. cylindracea* and surface associated bacteria is mutualistic. By the functional diversity data we can suggest that algae might exude those organic compounds that can be assimilated by bacteria and bacteria, on account of their metabolic diversity, might make both organic and inorganic carbon available to algae. The algal–bacterial association described here provides an identification tool that may be widely applicable for studying the marine invasion-bacterial association. Better spatial and temporal quantification of the here employed procedure may render this approach valuable and practical.

4.6. References

Aires T, Serrão EA, Kendrick G, Duarte CM, Arnaud-Haond S (2013) Invasion Is a Community Affair: Clandestine Followers in the Bacterial Community Associated to Green Algae,*Caulerpa racemosa*, Track the Invasion Source. PLoS ONE 8(7):e68429.

Ammerman JW, Azam F (1985) Bacterial 58-nucleotidase in 92 Y.A. Vetter, J.W. Deming aquatic ecosystems: a novel mechanism of phosphorus regeneration. Science 277:1338–1340.

Amon RMW, Benner R (1996) Bacterial utilization of different size classes of dissolved organic matter. Limnol Oceanogr 41:41–51.

Belton GS, Prud'homme van Reine, WF, Huisman JM, Draisma SGA, Gurgel CFD (2014) Resolving phenotypic plasticity and species designation in the morphology challenging *Caulerpa racemosa-peltata* complex (Caulerpaceae, Chlorophyta). J Phycol 50(1):32-54.

Benner R (2002) Chemical composition and reactivity. In: Biogeochemistry of Marine Dissolved Organic Matter, ed. DA Hansell, CA Carlson, pp. 59–90. San Diego, CA: Elsevier.

Bott TL, Kaplan L (1985) Bacterial biomass, metabolic state and activity in stream sediments: relation to environmental variables and multiple assay comparisons. Appl Environ Microbiol 50:508-522.

Burdige DJ, Zheng SL (1998) The biogeochemical cycling of dissolved organic nitrogen in estuarine sediments. Limnol Oceanogr 43:1796–1813.

Byers JE, Wright JT, Gribben PE (2010) Variable direct and indirect effects of a habitat modifying invasive species on mortality of native fauna. Ecology 91:1787-1798.

Cavallo RA, Rizzi C, Vozza T, Stabili L (1999) Viable heterotrophic bacteria in water and sediment in Mar Piccol' of Taranto (Ionian Sea, Italy). J Appl Microbiol 86(6):906-916.

Cebrian E, Linares C, Marchal C, Garrabou J (2012) Exploring the effects of invasive algae on the persistence of gorgonian populations. Biol Invasions 14:2647–2656.

Chattopadhyay K, Adhikari U, Lerouge P, Ray B (2007) Polysaccharides from *Caulerpa racemosa*: Purification and structural features. Carbohydr Polymer 68 :407-441.

Chisholm JRM, Jaubert JM, Giaccone G (1995) *Caulerpa taxifolia* in the Northwest Mediterranean: introduced species or migrant from the Red Sea. Comptes Rendus de l'Académie des Sciences, Series III, 318:1219–1226.

Cho BC, Azam F (1988) Major role of bacteria in biogeochemical fluxes in the ocean's interior. Nature 332:441–443.

Clarke KR (1993) Non-parametric multivariate analysis of changes in community structure. Aust J Ecol 18:117–143.

Clarke KR, Warwick RM (1994) Change in marine communities: an approach to statistical analysis and interpretation. Natural Environment Research Council, Plymouth, UK.

Clarke KR, Gorley RN (2006) PRIMER V6: User Manual/ Tutorial. Plymouth, UK: PRIMER-E.

Di Giacomo M, Paolino M, Silvestro D, Vigliotta G, Imperi F, Visca P, Alifano P, Parente D (2007) Microbial community structure and dynamics of dark fire-cured tobacco fermentation. Appl Environ Microbiol 73:825–837.

Dick WA, Tabatabai MA, Metting Jr FB (1992) Significance and potential uses of soil enzymes. In: Metting Jr FB (eds). Soil microbial ecology: applications in agricultural and environmental management. New York: Marcel Dekker, Inc. pp. 95-127.

Felline S, Caricato R, Cutignano A, Gorbi S, Lionetto MG, Mollo E, Regoli F, Terlizzi A (2012) Subtle effects of biological invasions: cellular and physiological responses of fish eating the exotic pest *Caulerpa racemosa* PLoS ONE 7:e38763. doi:10.1371/journal.pone.0038763.

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376.

Galtier N, Gouy M, Gautier C. (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12:543–538.

Goecke F, Labes A, Wiese J,Imhoff JF (2010) Chemical interactions between marine macroalgae and bacteria. Mar Ecol-Prog Ser 409 :267–300.

Gouy M, Guindon S, Gascuel O (2010) SeaView Version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27(2): 221-224.

Holmer M, Marbà N, Lamote M, Duarte CM (2009) Deterioration of Sediment Quality in Seagrass Meadows (*Posidonia oceanica*) Invaded by Macroalgae (*Caulerpa* sp.). Estuar Coast 32:456-466.

Hoppe HG (1991) Microbial extracellular enzyme activity: a new key parameter in aquatic ecology. In: RJ Chrost (ed) Microbial Enzymes in Aquatic Environments. Springer-Verlag, New York.

Hoppe HG, Ducklow H, Karrasch B (1993) Evidence for dependency of bacterial growth on enzyme hydrolysis of particulate organic matter in the mesopelagic ocean. Mar Ecol-Prog Ser 93:277–283.

Inagaki K, Arita M, Mizuskai S (1991) Properties of novel Ca super(2+)-activated protease from marine bacterium. In: Program and Abstracts, Second International Marine Biotechnology Conference IMBC '91.

Khotimchenko SV (1995) Fatty Acid Composition of Green Algae of the Genus *Caulerpa*. Bot Mar 38:509-512.

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120.

Klein J, Verlaque M (2008) The *Caulerpa racem*osa invasion: A critical review. Mar Pollut Bull 56: 205-225.

Lachnit T, Wahl M, Harder T (2010) Isolated thallus associated compounds from the macroalga *Fucus vesiculosus* mediate bacterial surface colonization in the field similar to that on the natural alga. Biofouling 26:247–255.

Lachnit T, Meske D, Wahl M, Harder T, Schmitz R (2011) Epibacterial community patterns on marine macroalgae are host-specific but temporally variable. Environ Microbio 13(3):655–665.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA 82:6955–6959.

Lee OO, Lai PY, Wu HX, Zhou XJ, Miao L, Wang H, Qian PY (2012) *Marinobacter xestospongiae* sp. nov., isolated from the marine sponge *Xestospongia testudinaria* collected from the Red Sea. Int J Syst Evol Microbiol 62(8):1980-5.

Lemke MJ, Churchill PF, Wetzel RG (1995) Effect of substrate and cell surface hydrophobicity on phosphate utilization in bacteria. Appl Environ Microbiol 61:913–919.

Yakimov M, Timmis KN, Golyshin PN (2007) Obligate oil-degrading marine bacteria. Current Opinion in Biotechnology 18(3):257–266.

Mancini I, Guella G, Defant A, Candenas M L, Armesto CP, Depentori D, Pietra F (1998) Polar metabolites of the tropical green seaweed *Caulerpa taxifolia* which is spreading in the Mediterranean Sea: glycoglycerolipids and stable enols (α/-keto esters). Helv Chim Acta 81:1681-1691.

Meusnier I, Olsen JL, Stam WT, Destombe C, Valero M (2001) Phylogenetic analyses of *Caulerpa taxifolia* (Chlorophyta) and of its associated bacterial microflora provide clues to the origin to the Mediterranean introduction. Mol Ecol 10:931–946.

Meyer-Reil LA (1991) Ecological aspects of enzyme activity in marine sediments. In: Chrost RJ (ed) Microbial Enzymes in Aquatic Environments. Springer-Verlag, New York.

Murrell MC, Hollibaugh JT (2000) Distribution and composition of dissolved and particulate organic carbon in northern San Francisco Bay during low flow conditions. Estuar Coast Shelf Sci 51:75–90.

Obernosterer I, Kraay G, De Kanitz E, Herndl GJ (1999) Concentrations of low molecular weight carboxylic acids and carbonyl compounds in Aegean Sea (Eastern Mediterranean) and the turnover of pyruvate. Aquat Microb Ecol 20:147–156.

Pinhassi J, Azam F, Hempha¨la¨J, Long RA, Martinez J, Zweifel UL, Hagstrom A (1999) Coupling between bacterioplankton species composition, population dynamics, and organic matter degradation. Aquat Microb Ecol 17:13–26.

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25:943–948.

Preston GM, Haubold B, Rainey PB (1998) Bacterial genomics and adaptation to life on plants: implications for the evolution of pathogenicity and symbiosis. Curr Opin Microbiol 1:589–597.

Provasoli L, Pintner IJ (1980) Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae). J Phycol 16:196–201.

Pullin MJ, Bertilsson S, Goldstone JV, Voeker BM (2004) Effects of sunlight andhydroxyl radical on dissolved organic matter: bacterial growth efficiency and production of carboxylic acids and other substrates. Limnol Oceanog 49:2011–2022.

Rajasulochana P, Krishnamoorthy P, Dhamotharan R (2013) Investigations on marine red and brown algae with respect to biochemical composition and nutraceuticals an overview. Int J Pharmacy Technol 4(4):5019-5027.

Rao D, Webb JS, Kjelleberg S (2006) Microbial colonization and competition on the marine alga Ulva australis. J Appl Microbiol. 72(8):5547–5555.

Rier ST, Stevenson RJ (2002) Effects of light, dissolved organic carbon, and inorganic nutrients on the relationship between algae and heterotrophic bacteria in stream periphyton. Hydrobiologia 489:179–184.

Robinson CH 2001. Cold adaptation in Arctic and Antarctic fungi. New Phytol 151:341–353.

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Sala MM, Arin L, Balagué V, Felipe J, Guadayol O, Vaqué D (2005) Functional diversity of bacterioplankton assemblages in western Antarctic seawaters during late spring. Mar Ecol-Prog Ser 292:13–21.

Sala MM, Estrada M, Gasol JM (2006) Seasonal changes in the functional diversity of bacterioplankton in contrasting coastal environments of the Northwest Mediterranean. Aquat Microb Ecol 44:1–9.

Sala MM, Terrado R, Lovejoy C, Unrein F, Pedrós-Alió C (2008) Metabolic diversity of heterotrophic bacterioplankton over winter and spring in the coastal Arctic Ocean. Environ Microbiol 10:942–949.

Salemi M, Vandamme AM. (2003) The phylogenetic handbook: a practical approach to DNA and protein phylogeny. Cambridge University Press, Cambridge, United Kingdom.

Shiba T (1992) The genus *Roseobacter*. In The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications (eds Starr MP, Stolp H, Trüpper HG, Balows A, Schelgel HG), pp. 202–206. Springer, Berlin.

Singh RP, Reddy CRK (2014) Seaweed–microbial interactions: key functions of seaweed-associated bacteria. FEMS Microb Ecol 88(2):213-238.

Smith DC, Simon M, Alldredge AL, Azam F (1992) Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. Nature. 359:139-142.

Sneed JM, Pohnert G (2011) The green macroalga Dictyosphaeria ocellata influences the structure of the bacterioplankton community through differential effects on individual bacterial phylotypes. FEMS Microbiol Ecol 75:242–254.

Sober E (1983) Parsimony in Systematics: Philosophical Issues. Annu Rev Ecol Evol Syst 14:335–357.

Stabili L, Gravili C, Tredici SM, Piraino S, Tala A, Boero F, Alifano P (2008) Epibiotic Vibrio luminous bacteria isolated from some Hydrozoa and Bryozoa species. Microb Ecol 56(4):625-636.

Svitil AL (1997) Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. Appl Environ Microbiol 63:408–413.

Truu M, Juhanson J, Truu J (2009) Microbial biomass, activity and community composition in constructed wetlands. Sci Total Environ 407:3958–3971.

Uchida M (1995) Enzyme activities of marine bacteria involved in Laminaria-thallus decomposition and the resulting sugar release. Mar Biol 123:639–644.

Van Hannen EJ, Mooij W, Van Agterveld MP, Gons HJ, Laanbroek HJ (1999) Detritus-dependent development of microbial community in an experimental system: qualitative analysis by denaturing gradient gel electrophoresis. Appl Environ Microbiol 65:2478–2484.

Verlaque M, Durand C, Huisman JM, Boudouresque CF, Le Parco Y (2003) On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). Eur J Phycol 38:325–339.

Vigliotta G, Tredici SM, Damiano F, Montinaro MR, Pulimeno R, di Summa R, Massardo DR, Gnoni GV, Alifano P (2005) Natural merodiploidy involving duplicated rpoB alleles affects secondary metabolism in a producer actinomycete. Mol Microbiol 55:396–412.

Vigliotta G, Nutricati E, Carata E, Tredici SM, De Stefano M, Pontieri P, Massardo DR, Prati MV, De Bellis L, Alifano P (2007) *Clonothrix fusca* Roze 1896, a filamentous, sheathed, methano- trophic gamma-proteobacterium. Appl Environ Microbiol 73:3556–3565.

Wahl M, Shahnaz L, Dobretsov S, Saha M, Symanowski F, David K, Lachnit T, Vasel M, Weinberger F (2010) Ecology of antifouling resistance in the bladder wrack Fucus vesiculosus: patterns of microfouling and antimicrobial protection.Mar Ecol-Prog Ser 411:33–48.

Weiss MS, Abele J, Weckesser W, Welte W, Schultz E, Schulz GE (1991) Molecular architecture and electrostatic properties of a bacterial porin. Science 254:1627–1630.

5. Functional biodiversity of the microbial community associated to the surface of *Caulerpa cylindracea* (Sonder) and potential effects on sediment biochemical composition

5.1 Abstract

Available data about the effects of biological invaders on prokariotic communities and related microbial processes are limited, particularly in marine environments. Biodiversity is strongly threatened by biological invasions and alien seaweeds have the capacity to change sediment biogeochemistry leading to profound alterations into the structure and functioning of ecosystems. *Caulerpa cylindracea* is an introduced invasive (alien) species widely distributed in all available habitats of the Mediterranean Sea. In this study, conducted in five sites across the central Mediterranean Sea, we compared, by the Biolog EcoPlate™, the metabolic patterns of the microbial communities associated to *C. cylindracea* surface, as well as to sediments colonized and uncolonized by the seaweed. Moreover, the biochemical composition of sediment organic matter was analysed in sites invaded and not-invaded by *C. cylindracea*. In all the examined sites, the microbial communities associated to the surface of *C. cylindracea* was able to utilize 16 out of 31 substrates and the microbial communities in the sediment colonized by the alga degraded 19 common substrates. By contrast, uncolonized by invasive alga sediments used only 16 of the 31 substrates. In addition, sediment organic matter concentration appears higher in seaweed colonized areas than seaweed uncolonized areas especially in terms of proteins and carbohydrates. Statistical analyses underlined significant differences among uncolonized areas and colonized areas leading to consider that the presence of the species *C. cylindracea* can alter the organic matter of the surrounding sediments as well as might host specific bacteria responsible for the degradation of compounds related to the presence of the seaweed itself.

5.2 Introduction

Biological invasions are considered one of the most crucial factors of global change inducing profound alterations into structure and functioning of ecosystems at several levels (Mckinney and Lockwood, 1999; Stachowicz et al., 2002; Stachowicz and Byrnes 2006, Galil, 2007). The introduction of non-indigenous species potentially habitat forming, may produce both direct and indirect effects on the sediment biogeochemistry (Neira et al., 2006; Lorenti et al., 2011). The ability of some alien species to trap sediments can support their spread, competitiveness and invasiveness (Airoldi and Cinelli, 1997; Airoldi, 2003). In turn, sedimentation of coastal areas is an important physical factor affecting biological assemblages through burial and smothering, supporting the spread of tolerant and opportunistic species (Irving and Connell, 2002a,b; Eriksson and Johansson, 2003; Balata et al., 2005). Among non-indigenous invasive species, the introduced tropical alga *Caulerpa cylindracea* (Sonder) (Belton et al., 2014), previously known as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman and Boudouresque (Verlaque et al., 2003) is now widely distributed in the Mediterranean basin (Klein and Verlaque, 2008) and is known to colonize all available habitats and substrates leading to profound structural and functional alterations of indigenous assemblages and fish metabolism (Holmer et al., 2009; Cebrian et al., 2012; Felline et al., 2012). *Caulerpa cylindracea* is able to enhance sediment accumulation, supporting algal turfs to the detriment of erect macroalgae, becoming, in this way, the main driver of ecological change (Bulleri and Benedetti-Cecchi, 2008; Bulleri et al., 2010). *Caulerpa cylindracea* is able to compact several layers of sediment up to 15 cm thick and to modify hydrodynamics near the seabed (Argyrou et al., 1999; Žuljević et al., 2003; Piazzi et al., 2007; Hendricks et al., 2010) increasing relative organic matter and sulphide pools (Casu et al., 2009, Holmer et al., 2009) and thus acting as ecosystem engineer (Wallentinus and Nyberg, 2007) with synergies between environmental and human-driven threats in subtidal habitat (Bulleri et al., 2011). The capacity of some introduced seaweeds to expand and become invasive could depend either on their intrinsic capacities to adapt to new environmental conditions (Facon et al., 2008; Klein and Verlaque, 2008) and on the associated microbial community, as recently demonstrated (Meusnier et al., 2001; Byers et al., 2010; Aires et al., 2013). Algae belonging to *Caulerpa* genus are associated to a complex and specific bacterial community as demonstrated by several studies reporting a similarity in the composition of the seaweed associated bacteria

independently from the seaweed location (Meusnier et al., 2001; Meusnier et al., 2002; Meusnier et al., 2004; Aires et al., 2013).

The transformation of complex organic macromolecules into low molecular weight compounds is due to heterotrophic prokaryotes producing extracellular enzymes so supplying a variety of suitable substrates (Arnosti, 2003; Danovaro et al., 2005). Heterotrophic prokaryotes strongly act in sediment organic matter degradation processes transferring energy to upper trophic levels (Danovaro et al., 2001). A recent study investigated the variability of biogeochemical variables in the sediments of Pelješac Peninsula (Croatia, Adriatic Sea) where *C. cylindracea* was present compared to uncolonized sites (Matijević et al., 2013); in particular, organic carbon, total nitrogen and total phosphorus contents were higher in the surface sediments at invaded sites. In spite of these recent efforts to understand underlying mechanism of *C. cylindracea* success, still limited information is available regarding the characteristics of sediments affected by the presence of invasive alga canopy (Piazzi et al., 2005; Piazzi et al., 2007; Holmer et al., 2009; Hendriks et al., 2010; Matijević et al., 2013).

In this study, we compared the metabolic patterns of the microbial communities associated to *C. cylindracea* surface, as well as to sediments invaded by *C. cylindracea* and to sediments were the invasive seaweed is absent by using the Biolog EcoPlate system (Biolog, Hayward, CA, USA). This assay represents a standardized method for determining bacterial oxidation of 31 ecologically relevant carbon substrates with a redox-sensitive tetrazolium indicator of microbial respiration (Truu et al., 2009). Further analyses on sediments were performed in order to investigate the effects of *C. cylindracea* invasion in this compartment. In particular, we compared the biochemical composition of sediment phytopigments (chlorophyll-a and pheopigment concentrations) and organic matter (proteins, carbohydrates and lipids) in *C. cylindracea* colonized and uncolonized areas.

5.3 Material and Methods

Sampling

The invasive seaweed *C. cylindracea* as well as the sediments colonized by the seaweed (AC) and the sediments were the invasive seaweed is absent (AU) were collected in September by SCUBA divers at shallow water (5-10 m depth) on a rocky substrate (Fig. 5.1) in five different sites: the Bay of Kotor, Montenegro (42°29'06.6"N, 18°41'28.6"E); Othonoi (Diapontine Islands) Greece (39° 50.257'N, 19° 24.037'E); the Marine Protected Area of Torre Guaceto (Brindisi), Italy (40°42'59.25"N, 17°48'5.12"E); Otranto (Lecce), Italy (40° 9'5.94"N, 18°29'27.16"E) and the Marine Protected Area of Porto Cesareo (Lecce), Italy (N 40°12.772', E017°48.218'). In each site (n=5), replicated (n=3) samples were collected and transferred to the laboratory under controlled temperature and processed within 4 h from sampling.

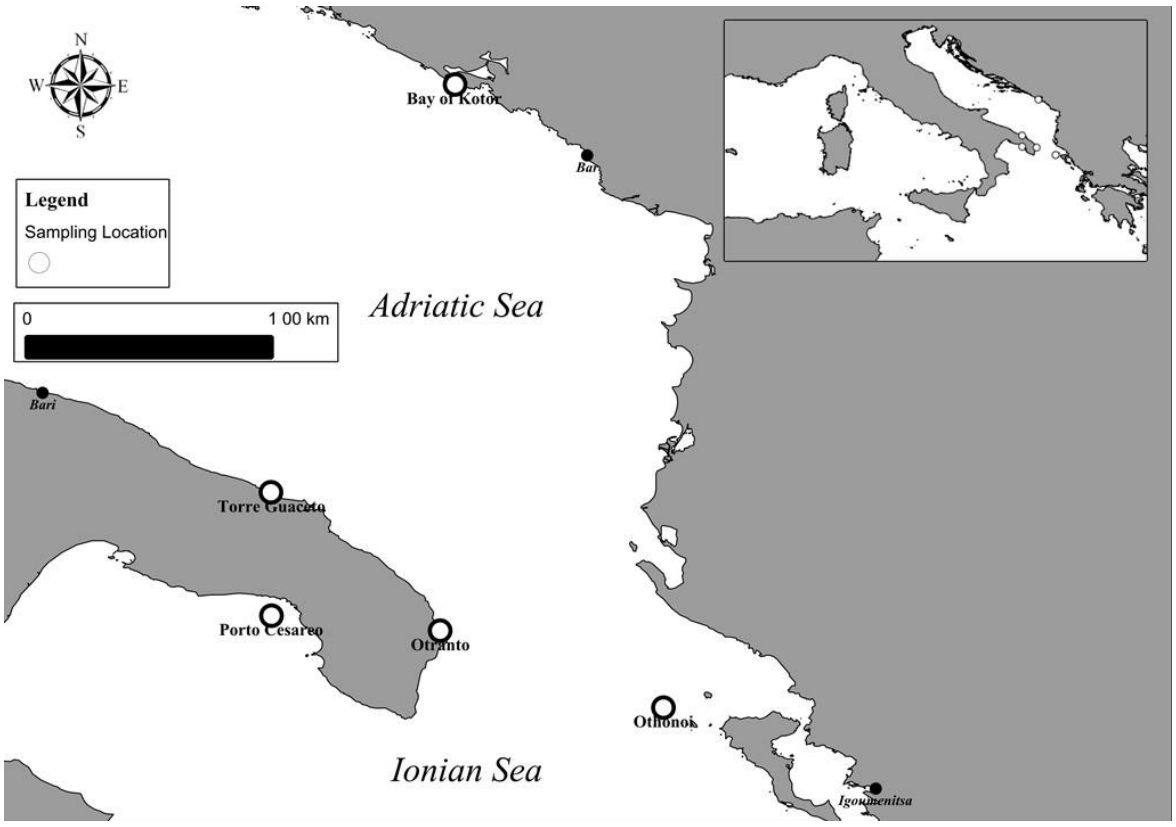


Figure 5.1. Map of the five sampling sites in the Mediterranean Sea.

BIOLOG ECO plate inoculation and incubation

In the laboratory, the alga (previously washed) and the sediments were suspended in sterile seawater and sonicated for three times (Branson Sonifier 2200, 60 W, 47 kHz for 1 min in an ice bath) to optimize surface bacteria detachment. The sonication was interrupted for 30 s every minute, during this time the samples were shaken manually. BIOLOG ECO plate (BIOLOG Inc., Hayward, Calif.) is a system made by a set of 31 substrates and one blank well in triplicate. In this work, three BIOLOG ECO plates were utilized for each algal sonicate, obtaining nine replicates. In each well a volume of 150 µL were inoculated and the BIOLOG ECO plates were incubated at 30 °C for 1 week. The optical density (OD) values were measured at a wavelength of 590 nm with a plate reader and the increase in optical density was recorded in each well, obtained by subtracting the optical density values at the beginning of incubation from the optical density at the end of incubation.

Biochemical composition of sediment organic matter

Chlorophyll-a and pheopigments analyses were carried out according to Danovaro et al. (2010). Pigments were extracted (12 h at 4 °C in the dark) from triplicate superficial (0-1cm) sediment samples, using 5ml of 90% acetone as the extractant. Extracts were analysed fluorometrically to estimate chlorophyll-a, and, after acidification with 200 µL of 0.1 N HCl, to estimate pheopigments concentrations. Concentrations are normalised to sediment dry weight and reported as µg g^{-1}. Total phytopigments were defined as the sum of chlorophyll-a and pheopigments (Pusceddu et al., 1999).

Proteins, carbohydrates and lipids sediment contents were analysed spectrophotometrically according to Danovaro et al. (2010) and concentrations expressed as bovine serum albumin, glucose and tripalmitine equivalents, respectively. For each biochemical assay, blanks were obtained using pre-combusted sediments (450 °C for 4 h). All analyses were performed on triplicate superficial (0-1cm) sediment samples. Carbohydrate, protein, and lipid concentrations were converted into carbon equivalents using the conversion factors 0.40, 0.49, and 0.75 mg C mg^{-1}, respectively, their sum was reported as biopolymeric organic carbon (Fabiano et al., 1995).

Statistical analysis

The differences among habitats on metabolic patterns of microbial communities and organic matter sediment composition in the five investigated sites, were tested in a multivariate context by a distance-based permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001; McArdle and Anderson, 2001). PERMANOVA was carried out separately on the biochemical composition of sediment organic matter and metabolic pattern of microbial community.

To test changes among the three habitats (algal surfaces, AS, sediments from areas colonized, AC, by *C. cylindracea*, and sediments from areas uncolonized, AU, by the seaweed) in the metabolic patterns of microbial communities, the experimental design consisted of two factors: Sites (Si, as random factor with 5 levels) and Habitats (Ha, as fixed factor with 3 levels orthogonal to Si) with n = 3 for each combination of factors. The analysis was based on Bray Curtis dissimilarities on untransformed data, using 4,999 random permutations of the appropriate units (Anderson and Braak, 2003).

The SIMPER procedure (Clarke, 1993) was used to identify the percentage contribution that each substrates made to the measures of the Bray-Curtis dissimilarity between the average of the AS, AC and AU. This analysis allowed identification of the most important substrates in discriminating AC from the AU.

To test differences on sediment organic matter among the two habitats (sediments from areas colonized AC by *C. cylindracea*, and sediments from areas uncolonized AU by the seaweed), the experimental design consisted of two factors: Sites (Si, as random factor with 5 levels) and Habitats (Ha, as fixed factor with 2 levels, presence/absence of *C. cylindracea,* orthogonal to Si). The analysis was based on Euclidean distances of previously normalized data, using 4,999 random permutations of the appropriate units (Anderson and Braak, 2003).

When significant differences were encountered ($p < 0.05$), post-hoc pairwise tests were also carried out, to ascertain in which sites the investigated variables were significantly different. Because of the restricted number of unique permutations in the pairwise tests, p values were obtained from Monte Carlo samplings. For metabolic patterns, canonical analysis of principal coordinates (CAP, Anderson and Willis, 2003; Anderson and Robinson, 2003) was performed for the factor of Habitat. For sediment organic matter, terms significant were examined using multidimensional scaling (MDS) plots of the fixed

factor (Habitat). The analyses were performed using the computer program PRIMER v. 6 (Clarke and Gorley, 2006).

5.4. Results

Bacterial metabolic profiles

The metabolic patterns of the bacterial communities associated to *C. cylindracea* surface (AS) and to the sediments from seaweed colonized areas (AC) and to the sediments from seaweed uncolonized areas (AU) were investigated using BIOLOG EcoPlates. Seaweed and sediment samples were collected in the five different sites of the Adriatic and Ionian Sea. The analyses of the metabolic profiles show a great ability of all the examined microbial communities to degrade several carbon sources (Table 5.1).

Table 5.1. Carbon sources degraded by the three microbial communities (AS, AC and AU) in the five sampled sites.

		Bay of Kotor			Othonoi Island			Otranto			Porto Cesareo			Torre Guaceto		
		Sediment from areas uncolonized (AU)	Sediment from areas colonized (AC)	*C. cylindracea* surface (AS)	Sediment from areas uncolonized (AU)	Sediment from areas colonized (AC)	*C. cylindracea* surface (AS)	Sediment from areas uncolonized (AU)	Sediment from areas colonized (AC)	*C. cylindracea* surface (AS)	Sediment from areas uncolonized (AU)	Sediment from areas colonized (AC)	*C. cylindracea* surface (AS)	Sediment from areas uncolonized (AU)	Sediment from areas colonized (AC)	*C. cylindracea* surface (AS)
Amino acids	L-Arginine	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
	L-Asparagine	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
	L-Phenylalanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	L-Serine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	L-Threonine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Glycyl-L-Glutamic Acid	+	+	+	-	-	-	+	+	-	-	-	-	+	+	-
	Phenylethyl-amine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Putrescine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrates	β-Methyl-D-Glucoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	i-Erythritol	+	+	-	-	-	-	+	+	-	-	-	-	+	+	-
	D-Mannitol	-	+	+	-	+	+	+	+	-	-	+	-	+	+	-
	N-Acetyl-D-Glucosamine	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Glucose-1-Phosphate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	α-D-Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D,L-α-Glycerol Phosphate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carboxylic Acid	D-Galactonic Acid γ-Lactone	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	Pyruvic Acid Methyl Ester	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D-Galacturonic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2-Hydroxy Benzoic Acid	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-
	4-Hydroxy Benzoic Acid	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+
	γ-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-Glucosaminic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Itaconic Acid	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	α-Ketobutyric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-Malic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Polymers	Tween 40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Tween 80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	α-Cyclodextrin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Glycogen	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-

In each site the greatest metabolic diversity was found in the sediments collected in the seaweed colonized areas (AC). In particular, the microbial communities of AC sediments are able to utilize 25, 20, 22, 19 and 21 carbon sources respectively in Bay of Kotor, Othonoi Island, Otranto, Porto Cesareo and Torre Guaceto. By contrast, the microbial communities of AU sediments degraded less carbon sources (19 in Bay of Kotor, 18 in Othonoi Island, 20 in Otranto, 16 in Porto Cesareo and 20 in Torre Guaceto). The bacterial communities associated to the algal surfaces (AS) are able to degrade 20 carbon sources in Bay of Kotor, 19 in Othonoi Island and 18 in Otranto, Torre Guaceto and Porto Cesareo. In all the examined samples the common metabolized substrates were 14: L-Phenylalanine, L-Serine, Phenylethyl-amine and Putrescine among amino acids; β-Methyl-D-Glucoside, D-Xylose, D-Cellobiose, Glucose-1-Phosphate, α-D-Lactose and D,L-α-Glycerol Phosphate among carbohydrates; Pyruvic Acid Methyl Ester and D-Galacturonic Acid among carboxylic acids; Tween 40 and Tween 80 among polymers. In addition to these carbon sources, all the microbial communities from sediments are able to degrade D-Galactonic Acid γ-Lactone and L-Arginine. Interestingly only the microbial communities of AS and AC degraded the 4-Hydroxy Benzoic Acid Carboxylic Acid.

Biochemical composition of sediment organic matter

Chlorophyll-a concentrations in the sediments showed great variability, and similar patterns are showed for pheopigment concentrations. The lowest sediment chorophyll-a concentration is observed in the uncolonized area (AU) of Othonoi and the highest one in the colonized area (AC) by *C. cylindracea* at the same site. The lowest sediment pheopigments concentration is observed in the uncolonized area (AU) of Torre Guaceto and the highest one in the colonized area (AC) by *C. cylindracea* at the same site. Total sediment phytopigments were defined as the sum of chlorophyll-a and pheopigments reported in Fig. 5.2.

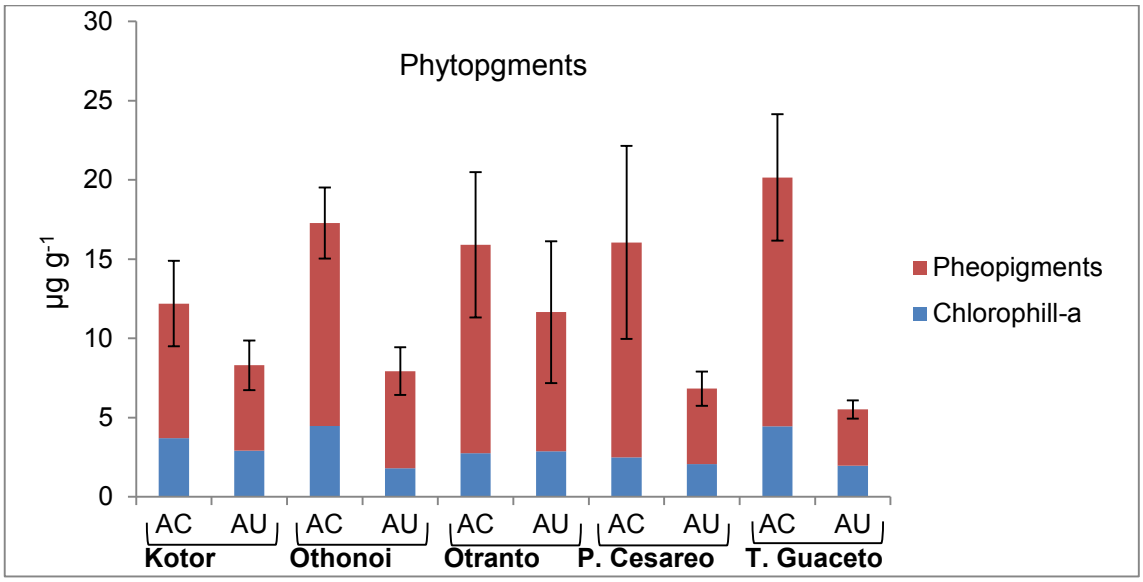


Figure 5.2. Phytopigment contents, spitted into Pheopigment and Chlorophyll-a concentrations in the sediment of all the 5 sites investigated (Standard error, n=3). AU=Presence of *C. cylindracea*, AU=Absence of *C. cylindracea*.

The lowest sediment protein concentration is observed in area uncolonized (AU) of Bay of Kotor and the highest one in area colonized (AC) by *C. cylindracea* of Porto Cesareo. The lowest sediment carbohydrate concentration is observed in area uncolonized (AU) of Bay of Kotor and the highest one in area colonized (AC) by *C. cylindracea* of Othonoi. The lowest sediment lipid concentration is observed in area uncolonized (AU) of Torre Guaceto and the highest one in area colonized (AC) by *C. cylindracea* at the same site. Carbon concentrations (BPC) were calculated as the sum of proteins, carbohydrate and lipid concentrations reported in Fig. 5.3.

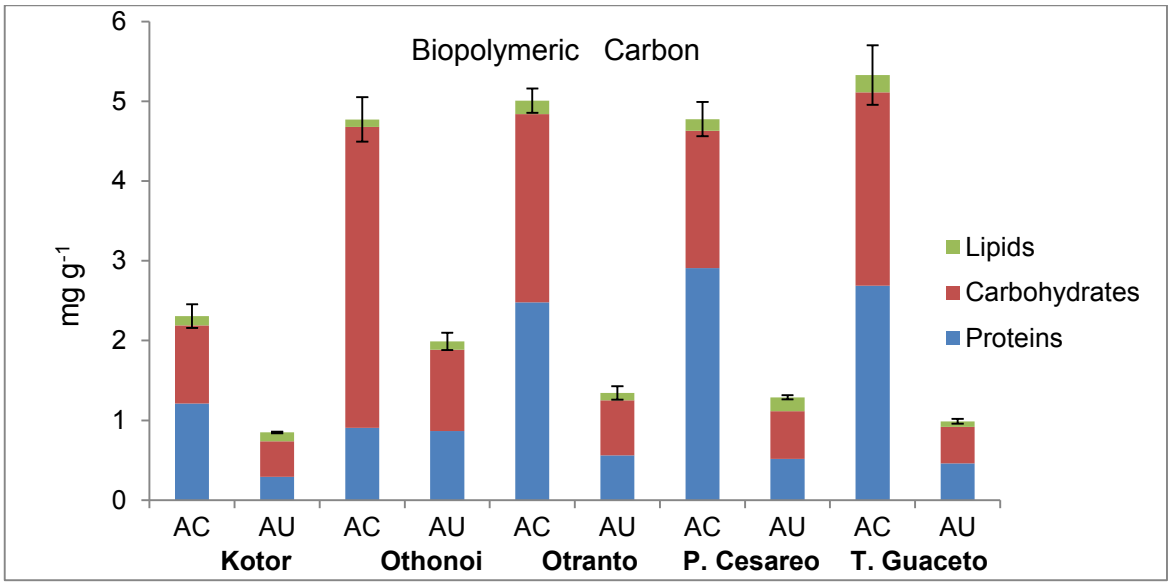


Figure 5.3. Biopolymeric Carbon concentrations, spitted into lipid, carbohydrate and protein concentrations in the sediment of all the 5 sites investigated (Standard error, n=3). AC=Presence of *C. cylindracea*, AU=Absence of *C. cylindracea*.

Statistical Analysis

The differences of bacterial metabolic patterns between examined habitats (algal surfaces, AS, sediments from areas colonized, AC, by *C. cylindracea*, and sediments from areas uncolonized, AU, by the seaweed) are significantly different from site to site as evidenced by the significant Si × Ha interaction term at the 0.0002 level (Table 5.2).

Table 5.2. Results of PERMANOVA testing for effects of habitats on the metabolic patterns of microbial communities in sites sampled.

Source	df	SS	MS	Pseudo-F	P(perm)	perms
Si	4	3125,9	781,48	17,989	0,0002	4983
Ha	2	2894,4	1447,2	4,6283	0,0002	4983
SixHa	8	2501,5	312,69	7,1976	0,0002	4969
Res	30	1303,3	43,443			
Total	44	9825,1				

Since significant differences were encountered ($p < 0.05$), post-hoc pairwise tests were also carried out, to ascertain in which sites the investigated variables show that differences were not consistent across sites. Monte Carlo p values were obtained, due to the restricted number of unique permutations in the pairwise tests. Pairwise comparisons of metabolic patterns among the 3 different habitats (algal surfaces AS, sediments from areas colonized AC by *C. cylindracea*, and sediments from areas uncolonized AU by the seaweed) within each Si × Ha combination revealed that differences were not consistent across sites (Table 5.3).

Table 5.3. PERMANOVA pairwise tests on metabolic patterns at the 3 different habitats within each Si × Ha combination.

PERMANOVA

PAIR-WISE TESTS

Term 'LoxHa' for pairs of levels of factor 'Habitat'
Within level 'BK' of factor 'Location'

Groups	T	perms	P(MC)
AC, AU	5,3573	10	0,0016
AC, AS	3,48	10	0,0034
AU, AS	4,3335	10	0,0026

Within level 'IO' of factor 'Location'

Groups	T	perms	P(MC)
AC, AU	2,9078	10	0,0106
AC, AS	2,7384	10	0,0108
AU, AS	4,5565	10	0,0034

Within level 'OT' of factor 'Location'

Groups	T	perms	P(MC)
AC, AU	2,3339	10	0,0462
AC, AS	3,2216	10	0,0072
AU, AS	4,1334	10	0,0032

Within level 'PC' of factor 'Location'

Groups	T	perms	P(MC)
AC, AU	3,1066	10	0,0074
AC, AS	2,2265	10	0,0224
AU, AS	2,7704	10	0,0076

Within level 'TG' of factor 'Location'

Groups	T	perms	P(MC)
AC, AU	2,7865	10	0,0092
AC, AS	3,3613	10	0,0054
AU, AS	4,0014	10	0,0024

SIMPER analysis showed the substrates that were important in differentiating metabolic patterns of microbial communities sampled in AC AU and AS (Table 5.4).

Table 5.4. Breakdown of average Bray-Curtis dissimilarity (untransformed data) values between AU and AC into contributions from the most important substrates.

SIMPER

	AC	AU	AS	AC vs. AU	AU vs. AS	AC vs. AS
Substrates	Av.Ab	Av.Ab.	Av.Ab.	Contrib%	Contrib%	Contrib%
4-Hydroxy Benzoic Acid	1,58	0,00	1,65	15,96	11,76	4,33
L-Serine	1,66	0,68	1,44	9,97	5,32	2,87
D-Mannitol	1,13	0,63	0,52	6,70	8,23	8,61
L-Asparagine	1,32	1,12	1,62	6,67	4,94	3,91
Glycyl-L-Glutamic Acid	0,42	1,07	0,38	5,91	5,41	2,85
Pyruvic Acid Methyl Ester	1,39	1,33	1,83	4,48	4,87	4,62
i-Erythritol	0,73	0,32	0,73	3,77	2,23	6,00
Putrescine	1,88	2,14	1,95	3,68	3,81	5,38
D-Galactonic Acid γ-Lactone	1,85	1,97	1,59	3,61	5,23	5,23
L-Phenylalanine	1,17	1,05	1,57	3,39	3,98	3,61
D-Galacturonic Acid	1,50	1,46	1,68	3,07	2,65	2,89
L-Arginine	1,53	1,67	0,81	2,99	6,54	6,48
N-Acetyl-D-Glucosamine	0,28	0,00	0,00	2,94	0,00	2,45
D-Cellobiose	1,45	1,48	1,68	2,93	2,84	3,26
Glycogen	0,29	0,00	0,00	2,89	0,00	2,45
Tween 80	1,77	1,59	1,87	2,73	2,06	2,54
α-D-Lactose	1,77	2,00	2,25	2,71	3,38	4,38
D,L-α-Glycerol Phosphate	1,69	1,71	2,03	2,57	4,29	5,26
Tween 40	1,46	1,42	1,54	2,51	2,60	3,34
Phenylethyl-amine	1,55	1,45	1,39	2,33	3,96	5,13
2-Hydroxy Benzoic Acid	0,20	0,00	0,53	2,25	4,22	2,99
β-Methyl-D-Glucoside	1,54	1,57	1,83	2,15	3,76	3,65
Glucose-1-Phosphate	1,48	1,48	1,78	1,52	3,18	3,43
Itaconic Acid	0,12	0,00	0,25	1,27	1,95	1,14
D-Xylose	1,60	1,58	1,82	1,01	2,80	3,22

CAP plot of fixed factor "Habitat" supports these results. The CAP achieved the highest allocation success (97.778%) using m = 7 principal coordinate axes, which themselves also explained 97.778% of the variation in the original dissimilarity matrix (Table 5.5).

Table 5.5. Results of leave-one-out allocation success from the canonical analysis of principal coordinates (CAP). The analysis was done using the first m = 7 principal coordinate axes (explaining 97.778% of the variation in the original dissimilarity matrix) based on the Bray-Curtis dissimilarities on untransformed data. Results of PERMANOVA testing for effects of habitats on the metabolic patterns of microbial communities in sites sampled.

Cross Validation
Leave-one-out Allocation of Observations to Groups
(for the choice of m: 7)

	Classified				
Orig. group	Y	N	A	Total	%correct
Y	14	0	1	15	93,333
N	0	15	0	15	100
A	0	0	15	15	100

Total correct: 44/45 (97,778%)
Mis-classification error: 2,222%

The 2 canonical axes (the number required to distinguish 3 groups in multivariate space) had very high canonical correlations with the multivariate assemblages. Pearson correlations (>0.75) show 4-hydroxy benzoic acid and L-serine mainly characterized the microbial communities on *C. cylindracea* surface (AS) and in sediments from areas colonized (AC) by the macroalga (Fig. 5.4).

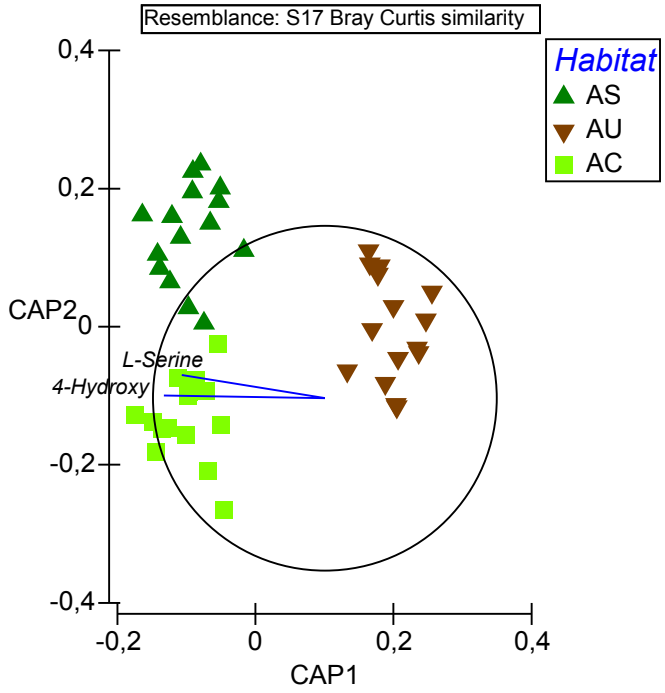


Figure 5.4. Canonical analysis of principal coordinates (CAP) plot showing canonical axes that best discriminate metabolic patterns at the 3 different habitats and correlations of carbon sources variables with the canonical axes.

Effects of presence of *C. cylindracea* on organic matter varied from site to site as highlighted by the significant Si × Ha interaction term at the 0.0054 (Table 5.6).

Table 5.6. Results of PERMANOVA testing for effects of habitats on the sediment organic matter of microbial communities in sites sampled.

Source	df	SS	MS	Pseudo-F	P(perm)	perms
Si	4	16,029	4,0071	2,2028	0,0184	4984
Ha	1	43,669	43,669	8,7692	0,0184	3566
SixHa	4	19,919	4,9798	2,7374	0,0054	4979
Res	20	36,383	1,8192			
Total	29	116				

Pairwise comparisons of organic matter at the 2 areas within each site revealed they were statistically significant among habitats in all sites, with the exception of Bay of Kotor where no differences were highlighted (Table 5.7).

Table 5.7. PERMANOVA pairwise tests on sediment organic matter at the different habitats within each Si × Ha combination.

PERMANOVA

PAIR-WISE TESTS

Term 'SixHa' for pairs of levels of factor 'Habitat'

Within level 'BK' of factor 'Location'

Groups	t	perms	P(MC)
AS,AU	1,4174	10	0,1702

Within level 'IO' of factor 'Location'

Groups	t	perms	P(MC)
AS,AU	2,9868	10	0,0186

Within level 'OT' of factor 'Location'

Groups	t	perms	P(MC)
AS,AU	2,5116	10	0,021

Within level 'PC' of factor 'Location'

Groups	t	perms	P(MC)
AS,AU	2,2993	10	0,0422

Within level 'TG' of factor 'Location'

Groups	t	perms	P(MC)
AS,AU	3,0924	10	0,021

MDS plots of fixed factor "Habitat" (Fig. 5.5), showed a separation among uncolonized (AU) vs. colonized (AC) areas, except for Bay of Kotor site, supporting general results.

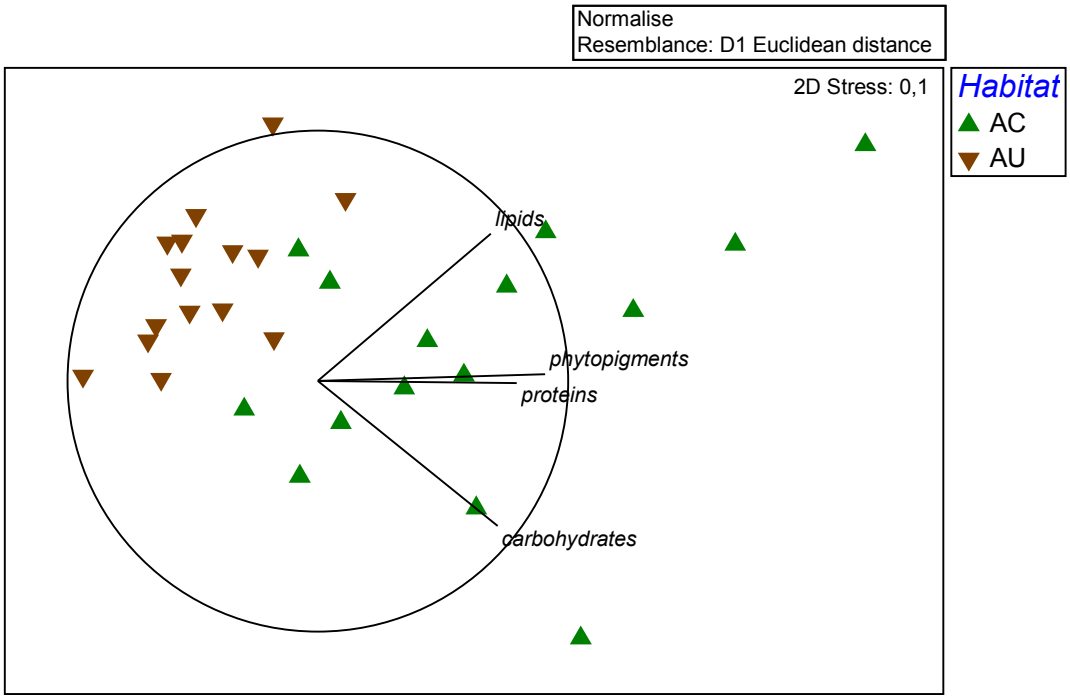


Figure 5.5. MDS plot showing 2 different habitats (invaded vs. not invaded) and Person correlations (>0.75) of carbon sources variables.

5.5. Discussion

One of possible effects of invasive species is to modify the recipient community so that subsequent changes in decomposition and/or nutrient cycling occur (Windham, 2001) affecting the organic matter, determining specific chemical composition and altering the decomposer microbial community. The consequences of an altered organic matter regime or altered microbial community may be manifested as changes in the rate of decomposition or the ability of the microbial community to degrade organic matter. In this framework, in the present work we compared the metabolic patterns of the microbial communities associated to three different habitats (*C. cylindracea* surface, sediments colonized by the seaweed and sediments uncolonized by the seaweed) in five sites across the central Mediterranean Sea, founding significant differences among them. Interestingly, among the carbon sources tested by using the Biolog EcoPlate system (Biolog, Hayward, CA, USA), only microbial communities associated to *C. cylindracea*

surfaces and sediments was able to degrade 4-hydroxy benzoic acid. Furthermore, in the five selected sites we compared the sediment biochemical composition observing higher concentration of organic matter in areas featured by the presence of the seaweed than areas where *C. cylindracea* was absent.

The genus *Caulerpa* is well-known to harbor endosymbiotic and epiphytic bacterial community, probably linked to a variety of metabolic functions, such as nitrogen fixation and production of various toxic compounds (Burr and West, 1970; Turner and Friedman, 1974; Dawes and Lohr, 1978; Provasoli and Pintner, 1980; Shiba, 1992; Weidner et al., 1996). Seaweed-associated bacteria could exchange signals with their algal hosts and metabolize algal derived compounds and synthesize algal hormones (Preston et al., 1998). The analysis of the bacterial metabolic profiles associated to *C. cylindracea* surface and the sediments revealed a great ability of the bacterial communities to degrade several carbon sources. In all the examined sites the microbial communities associated to the surface of *C. cylindracea* were able to utilize 16 common substrates. In addition, in each site, the bacterial metabolic patterns of the communities in seaweed colonized sediments are able to degrade a higher number of substrates than the microbial communities from seaweed uncolonized sediments. Statistical analysis performed on the metabolic patterns of the microbial communities highlighted significant differences among the algal surface, the seaweed colonized sediment and the seaweed uncolonized sediment. SIMPER analysis revealed the important substrates driving the differences between the microbial metabolic patterns of the seaweed colonized sediment from those of seaweed uncolonized sediment. These included some amino acids, carbohydrates and carboxylic acids. In addition, the analysis of biochemical composition of sediment organic matter in *C. cylindracea* colonized and uncolonized areas showed significant differences in all the examined sites, except for Bay of Kotor suggesting that the macroalga can potentially change the sediment composition presumably furnishing more proteins and carbohydrates. Marine algae, indeed, are an important source of organic matter in coastal waters. Proteins and labile carbohydrates mostly derive from algae and represent an important organic source for marine sediments. Marine algae contain various carbohydrates, and in addition, high protein content has been reported (Murugaiyan and Narasimman, 2013). Recently, it has been shown that the biochemical composition of *C. cylindracea* includes 17.8-18.4% of protein, 33-41% carbohydrate, 9.8% of lipids, 64.9% of fiber and 7-19% of ash (Pereira, 2011). We have observed higher protein and

carbohydrate concentrations in seaweed colonized sediment than seaweed uncolonized sediment. L-serine and L-asparagine resulted by Biolog EcoPlate™ as the degraded carbon sources that mostly contribute to distinguish the microbial community colonized by *C. cylindracea* sediments from the uncolonized sediments. L-serine is an amino acid that plays an important catalytic role in the functions of numerous enzymes; it have been found naturally in seaweeds as well as in Caulerpaceae (Ratana-arporn and Chirapart, 2006). The degradation of L serine by the bacterial community present in *C. cylindracea* colonized sediments suggests that the seaweed could furnish this carbon source to bacterial communities living in the same environment. It is known that several species belong to genus *Shewanella* are able to degrade l-serine and this genus was found on *C. cylindracea* surface (Rizzo et al., in preparation, see Chapter 2). L- asparagine is a component of living organisms utilized by some marine luminous bacteria belonging to *Vibrio* and *Photobacterium* genera to produce l-asparaginase (Ramaiah and Chandramohan, 1992). Interestingly both these two genera were found on *C. cylindracea* surface (Rizzo et al., in preparation; see Chapter 2). On account of these results we can hypothesize that algal host is firstly crucial to bacterial community functions and, secondly, to affects the ecology and the patterns of degradation of the habitats in which it is present.

In the marine environment carbohydrates are considered the most labile fractions of organic matter (Benner et al., 1992; Burdige and Zheng, 1998; Murrell and Hollibaugh, 2000). Amino acids and carbohydrates are considered an important group of cell constituents in algae and *C. cylindracea* could provide several carbon sources to microbial communities. Among the carbohydrates tested by the Biolog EcoPlate™, D-mannitol resulted as the degraded carbon sources that mostly contribute to distinguish the microbial community of *C. cylindracea* colonized sediments from seaweed uncolonized sediment. D-mannitol is a common carbohydrate constituent of marine algae (Fong and Mann, 1980) and presumably the presence of the alga is responsible for the higher concentration in the seaweed colonized sediments.

Carboxylic acids are considered important part of the labile pool of organic matter in several aquatic environments (Obernosterer et al., 1999; Pullin et al., 2004; Sala et al., 2006). Noteworthy, 4-hydroxy benzoic acid is a carboxylic acid degraded only by the microbial community associated to the surface and to the sediments colonized by *C. cylindracea*. The hydroxybenzoic acids are important intermediate metabolites in

degradation of various aromatic compounds due to some bacteria including the genus *Bacillus* (Karegoudar and Kim, 2000). Interestingly bacteria belonging to this genus were isolated from *C. cylindracea* surface (Rizzo et al., in preparation, see Chapter 2). 4-hydroxy benzoic acid is a phenolic compound also synthesized by *Posidonia oceanica* leaves and supposed to be involved in the protection against pathogens as well as in the allelopathy by reducing the growth of competing plants (Cariello and Zanetti, 1979; Kuo and Mc Comb, 1989; Taiz and Zeiger, 1998; Tavares-Colpas et al., 2003). Some authors have suggested that phenolic compounds with medium polarity are synthesized by *C. cylindracea* and exert an antioxidative activity of (Li et al., 2012). Parabens, esters of 4-hydroxy benzoic acid are commonly used as antimicrobial, ant-fungicidal and antioxidants agents in the cosmetic and pharmaceutical industries (Radovan et al., 2008) released continuously into the aquatic environment (Benijts et al., 2004). On the light of our results further studies will be performed to evaluate whether the bacilli isolated from *C. cylindracea* surface are actually responsible for the 4-hydroxy benzoic acid degradation.

Among the polymers tested by the Biolog EcoPlate™, Tween 40 and Tween 80 were utilized by all the sampled microbial communities. Thus, on account of our results the degradation of these polymers do not represent a key factor on discriminating *C. cylindracea* surface as well as seaweed colonized and uncolonized sediment. On the other hand their utilization was already been reported for other microbial communities (Robinson, 2001; Sala et al., 2005; Sala et al., 2008).

In all explored sediments, the contribution of primary organic matter (as total phytopigment concentrations) to biopolymeric carbon was very low, indicating that the investigated ecosystems were largely dominated by detrital or heterotrophic organic matter. All experimented sites are oligotrophic areas, nevertheless Brindisi and Otranto ports, as well as other parts of Adriatic Sea, are under the influence of anthropogenic activities present on the coastline (Dell'Anno et al., 2002; Dautovic et al., 2012). Also the Bay of Kotor is under recent influence of the increasing urbanization of the coastal zone without a developmental strategy and with the disrespect of existing regulations, even though natural eutrophication is still dominant over that of anthropogenic eutrophication (Krivokapić et al., 2011). Gennaro and Piazzi (2011) have detected significant interaction between nutritional increase and *C. cylindracea* invasion: the effects of *C. cylindracea* invasion on native macroalgal assemblages were amplified by nutrient enrichment

suggesting synergism between effects of anthropogenic impacts with severe consequences on the integrity of marine ecosystems.

In conclusion, our results indicate that the invasive algal species *C. cylindracea* can modify the organic matter of the surrounding sediments as well as might host specific bacteria responsible for the degradation of compounds related to the presence of the seaweed itself. Obviously not all the aspects of the mechanisms by which the seaweed influence the surface associated bacterial community were elucidated in this study and warrants further investigation demonstrating that in marine systems, more research should focus on the effects of invaders on the microbial component of the recipient community as these microbial communities may strongly affect broader ecosystem processes.

5.6. References

Aires T, Serrão EA, Kendrick G, Duarte CM, Arnaud-Haond S (2013) Invasion Is a Community Affair: Clandestine Followers in the Bacterial Community Associated to Green Algae,*Caulerpa racemosa*, Track the Invasion Source. PLoS ONE 8(7): e68429. doi:10.1371/journal.pone.0068429

Airoldi L, Cinelli F (1997) Effect of sedimentation on subtidal macroalgal assemblages: an experimental study from a Mediterranean rocky shore. J Exp Mar Biol Ecol 215:271-290.

Airoldi L (2003) The effects of sedimentation on rocky coast assemblages. Oceanogr Mar Biol Ann Rev 41:161–236.

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Aust Ecol 26:32–46.

Anderson M, Braak CT (2003) Permutation tests for multi-factorial analysis of variance. J Stat Comput Sim 73(2):85-113.

Anderson MJ, Willis TJ (2003) Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. Ecology 84:511–525.

Anderson MJ, Robinson J (2003) Generalised discriminant analysis based on distances. Aust NZ J Stat 45:301–318.

Argyrou M, Demetropoulos A, Hadjichristophorou M (1999) Expansion of the macroalga *Caulerpa racemosa* and changes in softbottom macrofaunal assemblages in Moni Bay, Cyprus. Oceanol Acta 22:517–528.

Arnosti C (2003) Microbial extracellular enzymes and their role in dissolved organic matter cycling, In: Sinsabaugh RL eds. Aquatic ecosystems: interactivity of dissolved organic matter San Diego: Academic Press 315–342.

Balata D, Piazzi L, Cecchi E, Cinelli. F (2005) Variability of Mediterranean coralligenous assemblages subject to local variation in sediment deposits. Mar Env Res 60:403-421.

Belton GS, Prud'homme van Reine, WF, Huisman JM, Draisma SGA, Gurgel CFD (2014) Resolving phenotypic plasticity and species designation in the morphology challenging *Caulerpa racemosa-peltata* complex (Caulerpaceae, Chlorophyta). J Phycol 50(1):32-54.

Benijts T, Dams R, Lambert W, De Leenheer A (2004) Countering matrix effects in environmental liquid chromatography–electrospray ionization tandem mass spectrometry water analysis for endocrine disrupting chemicals. Journal Chromatogr A 1029(1):153-159.

Benner R, Pakulski JD, McCarty M, Hedges JI, Hatcher PG (1992) Bulk chemical characteristics of dissolved organic matter in the ocean. Science 255:1561-1564.

Bulleri F, Benedetti-Cecchi L (2008) Facilitation of the introduced green alga, *Caulerpa racemosa*, by resident algal turfs: experimental evaluation of underlying mechanisms. Mar Ecol-Prog Ser 364:77–86.

Bulleri F, Balata D, Bertocci I, Tamburello L, Benedetti-Cecchi L (2010) The seaweed *Caulerpa racemosa* on Mediterranean rocky reefs: from passenger to driver of ecological change. Ecology 91(8):2205–2212.

Bulleri F, Alestra T, Ceccherelli G, Tamburello L, Pinna S, Sechi N, Benedetti Cecchi L (2011) Determinants of *Caulerpa racemosa* distribution in the north-western Mediterranean. Mar Ecol-Prog Ser. 431:55-67.

Burdige DJ, Zheng SL (1998) The biogeochemical cycling of dissolved organic nitrogen in estuarine sediments. Limnol Oceanogr 43:1796–1813.

Burr FA, West JA (1970) Light and electron microscope observations on the vegetative and reproductive structures of *Bryopsis hypnoides*. Phycologia 9:17–37.

Byers JE, Wright JT, Gribben PE (2010) Variable direct and indirect effects of a habitat modifying invasive species on mortality of native fauna. Ecology 91:1787-1798. doi:10.1890/09-0712.1. PubMed: 20583719.

Cariello L, Zanetti L (1979) Distribution of chicoric acid during leaf development of *Posidonia oceanica*. Bot Mar 22(6):359-360.

Casu D, Ceccherelli G, Sechi N, Rumolo P, Sara` G (2009) *Caulerpa racemosa* var. *cylindracea* as a potential source of organic matter for benthic consumers: evidences from a stable isotope analysis. Aquat Ecol 43:1023–1029.

Cebrian E, Linares C, Marchal C, Garrabou J (2012) Exploring the effects of invasive algae on the persistence of gorgonian populations. Biol Invasions 14:2647–2656.

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18:117-143.

Clarke KR, Gorley RN (2006) PRIMER V6: User Manual/ Tutorial. Plymouth, UK: PRIMER-E.

Danovaro R, Armeni M, Dell'Anno A, Fabiano M, Manini E, Marrale D, Pusceddu A, Vanucci S (2001) Small-scale distribution of bacteria, enzymatic activities, and organic matter in coastal sediments. Microb Ecol 42(2):177-185.

Danovaro R, Armeni M, Luna GM, Corinaldesi C, Dell'Anno A, Ferrari CR, Fiordelmondo C, Gambi C, Gismondi M, Manini E, Mecozzi M, Perrone FM, Pusceddu A, Giani M (2005) Exo-enzymatic activities and dissolved organic pools in relation with mucilage development in the northern Adriatic Sea. Sci Total Environ 353:189–203.

Danovaro R (2010) Methods for the Study of Deep-Sea Sediments, their Functioning and Biodiversity. CRC Press: Boca Raton.

Dautović J, Strmečki S, Pestorić B, Vojvodić V, Plavšić M, Krivokapić S, Ćosović B (2012) Organic matter in the karstic enclosed bay (Boka Kotorska Bay, south Adriatic Sea). Influence of freshwater input. Fresen Environ Bull 21(4):995-1006.

Dawes CJ, Lohr CA (1978) Cytoplasmic organization and endosymbiotic bacteria in the growing points of *Caulerpa prolifera*. Rev Algol 13:309–314.

Dell'Anno A, Mei ML, Pusceddu A, Danovaro R (2002) Assessing the trophic state and eutrophication of coastal marine systems: a new approach based on the biochemical composition of sediment organic matter. Mar Pollut Bull 44(7):611-622.

Eriksson BK, Johansson G (2003) Sedimentation reduces recruitment success of *Fucus vesciculosus* (Phaeophyceae) in the Baltic Sea. Eur J Phycol 38:217-222.

Fabiano, M., Danovaro, R. Fraschetti, S (1995) A three-year time series of elemental and biochemical composition of organic matter in subtidal sandy sediments of the Ligurian Sea (northwest Mediterranean). Cont Shelf Res 15:1453-1469.

Facon B, Pointier JP, Jarne P, Sarda V, David P (2008) High genetic variance in life-history strategies within invasive populations by way of multiple introductions. Curr Biol 18:363–367. doi:10.1016/j.cub. 2008.01.063. PubMed: 18334202.

Felline S, Caricato R, Cutignano A, Gorbi S, Lionetto MG, Mollo E, Regoli F, Terlizzi A (2012) Subtle effects of biological invasions: cellular and physiological responses of fish eating the exotic pest *Caulerpa racemosa* PLoS ONE 7:e38763. doi:10.1371/journal.pone.0038763

Fong W, Mann KH (1980) Role of gut flora in the transfer of amino acids through a marine food chain. Can J Fish Aquat Sci 37(1):88-96.

Galil BS (2007) Loss or gain? Invasive aliens and biodiversity in the Mediterranean Sea. Mar Poll Bull 55:314–322.

Gennaro P, Piazzi L (2011) Synergism between two anthropic impacts: *Caulerpa racemosa* var. *cylindracea* invasion and seawater nutrient enrichment. Mar Ecol-Prog Ser 427:59-70.

Hendricks IE, Bouma TJ, Morris EP, Duarte CM (2010) Effects of seagrasses and algae of the *Caulerpa* family on hydrodynamics and particle-trapping rates. Mar Biol 157:473–481.

Holmer M, Marbà N, Lamote M, Duarte CM (2009) Deterioration of Sediment Quality in Seagrass Meadows (*Posidonia oceanica*) Invaded by Macroalgae (*Caulerpa* sp.). Estuar Coast 32:456-466.

Irving AD, Connell SD (2002a) Sedimentation and light penetration interact to maintain heterogeneity of subtidal habitats: algal versus invertebrate dominated assemblages. Mar Ecol-Prog Ser 245:83-91.

Irving AD, Connell SD (2002b) Interactive effects of sedimentation and microtopography on the abundance of subtidal turf-forming algae. Phycologia 41:517-522.

Karegoudar TB, Kim CK (2000) Microbial degradation of monohydroxybenzoic acids. J Microbiol Seoul 38(2):53-61.

Klein J, Verlaque M (2008) The *Caulerpa racemosa* invasion: A critical review. Mar Pollut Bull 56:205-225.

Krivokapić S, Pestorić B, Bosak S, Kuspilić G, Wexels-Riser C (2011) Trophic state of Boka Kotorska Bay (South-Eastern Adriatic Sea). Fresen Environ Bull 20(8):1960-1969.

Kuo J, Mc Comb A-J (1989) Seagrass taxonomy, structure and development. In: Biology of seagrass, Aquatic Plant studies Edited by: Larkum AWD, Mc Comb AJ, Sheperd SA. Elsevier publ 6-73.

Li Z, Wang B, Zhang Q, Qu Y, Xu H, Li G (2012) Preparation and antioxidant property of extract and semipurified fractions of *Caulerpa racemosa*. J Appl Phycol 24(6):1527-1536.

Lorenti M, Gambi MC, Guglielmo R, Patti FP, Scipione MB, Zupo V, Buia MC (2011) Soft bottom macrofaunal assemblages in the Gulf of Salerno, Tyrrhenian Sea, Italy, affected by the invasive seaweed *Caulerpa racemosa* var. *cylindracea*. Mar Ecol 32(3):320-334.

Matijevic S, Bogner M, Bojanić N, Žuljević A, Despalatović M, Antolić B, Nikolić V, Bilić J (2013) Biogeochemical characteristics of sediments under the canopy of invasive alga *Caulerpa racemosa* var. cylindracea (Peljesac Peninsula, Adriatic Sea). Fresen Environ Bull 22:3030-3040.

McArdle BH, Anderson MJ (2001) Fitting multivariate models to community data: A comment on distance-based redundancy analysis. Ecology 82:290–297.

McKinney ML, Lockwood JL (1999) Biotic Homogenization: A few winners replacing many losers in the next mass extinction. Trends Ecol Evol 14:450–453. doi:10.1016/S0169-5347(99)01679-1. PubMed:10511724.

Meusnier I, Olsen JL, Stam WT, Destombe C, Valero M (2001) Phylogenetic analyses of *Caulerpa taxifolia* (Chlorophyta) and of its associated bacterial microflora provide clues to the origin to the Mediterranean introduction. Mol Ecol 10:931–946. doi:10.1046/j. 1365-294X.2001.01245.x. PubMed: 11348502.

Meusnier I, Valero M, Destombe C, Godé C, Desmarais E, Bonhomme F, Stam WT, Olsen JL (2002) PCR-SSCP analyses of nuclear and chloroplast DNA provide evidence for recombination, multiple introductions and nascent speciation in the *Caulerpa taxifolia* complex. Mol Ecol 11:2317–2325. PubMed: 12406242.

Meusnier I, Valero M, Olsen JL, Stam WT (2004) Analysis of rDNA ITS1 indels in *Caulerpa taxifolia* (Chlorophyta) ports a derived, incipient species status for the invasive strain. Eur J Phycol 39:83–92. doi:10.1080/09670260310001646531.

Murrell MC, Hollibaugh JT (2000) Distribution and composition of dissolved and particulate organic carbon in northern San Francisco Bay during low flow conditions. Estuar Coast Shelf Sci 51:75–90.

Murugaiyan K, Narasimman S (2013) Biochemical and Mineral contents of selected Green Seaweeds from Gulf of Mannar Coastal region, TamilNadu, India.

Neira C, Grosholz ED, Levin LA, Blake R (2006) Mechanisms generating modification of benthos following tidal flat invasion by a *Spartina hybrid*. Ecol Appl 16:1391–1404.

Obernosterer I, Kraay G, De Kanitz E, Herndl GJ (1999) Concentrations of low molecular weight carboxylic acids and carbonyl compounds in Aegean Sea (Eastern Mediterranean) and the turnover of pyruvate. Aquat Microb Ecol 20:147–156.

Pereira L (2011) A review of the nutrient composition of selected edible seaweeds. Seaweed: Ecology, Nutrient Composition and Medicinal Uses 15-47.

Piazzi L, Balata D, Ceccherelli G, Cinelli F (2005) Interactive effect of sedimentation and *Caulerpa racemosa* var. *cylindracea* invasion on macroalgal assemblages in the Mediterranean Sea. Estuar Coast Shelf Sci 64:467-474.

Piazzi L, Balata D, Foresi L, Cristaudo C, Cinelli F (2007) Sediment as a constituent of Mediterranean benthic communities dominated by *Caulerpa racemosa* var. *cylindracea*. Sci Mar 71(1):129–135.

Preston GM, Haubold B, Rainey PB (1998) Bacterial genomics and adaptation to life on plants: implications for the evolution of pathogenicity and symbiosis. Curr Opin Microbiol 1:589–597.

Provasoli L, Pintner IJ (1980) Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae). J Phycol 16:196–201.

Pullin MJ, Bertilsson S, Goldstone JV, Voeker BM (2004) Effects of sunlight andhydroxyl radical on dissolved organic matter: bacterial growth efficiency and production of carboxylic acids and other substrates. Limnol Oceanog 49:2011–2022.

Pusceddu A, Sara G, Armeni M, Fabiano M, Mazzola A (1999) Seasonal and spatial changes in the sediment organic matter of a semi-enclosed marine system (W-Mediterranean Sea). Hydrobiologia 397(12):59-70.

Radovan C, Cinghită D, Manea F, Mincea M, Cofan C, Ostafe V (2008) Electrochemical sensing and assessment of parabens in hydro-alcoholic solutions and water using a boron-doped diamond electrode. Sensors 8(7):4330-4349.

Ramaiah N, Chandramohan D (1992) Production of L-asparaginase by the marine luminous bacteria. Indian J Mar Sci 21:212-214.

Ratana-arporn P, Chirapart A (2006) Nutritional evaluation of tropical green seaweeds *Caulerpa lentillifera* and *Ulva reticulata*. Kasetsart J 40:75-83.

Rizzo L, Fraschetti S, Alifano P, Pizzolante G, Stabili L (in prep.) Heterotrophic bacteria associated to the surface of *Caulerpa cylindracea* in the Mediterranean Sea.

Robinson CH (2001) Cold adaptation in Arctic and Antarctic fungi. New Phytol 151:341–353.

Sala MM, Arin L, Balagué V, Felipe J, Guadayol O, Vaqué D (2005) Functional diversity of bacterioplankton assemblages in western Antarctic seawaters during late spring. Mar Ecol-Prog Ser 292:13–21.

Sala MM, Estrada M, Gasol JM (2006) Seasonal changes in the functional diversity of bacterioplankton in contrasting coastal environments of the Northwest Mediterranean. Aquat Microb Ecol 44:1–9.

Sala MM, Terrado R, Lovejoy C, Unrein F, Pedrós-Alió C (2008) Metabolic diversity of heterotrophic bacterioplankton over winter and spring in the coastal Arctic Ocean. Environ Microbiol 10:942–949.

Shiba T (1992) The genus *Roseobacter*. In *The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications* (eds Starr MP, Stolp H, Trüpper HG, Balows A, Schelgel HG), pp. 202–206. Springer, Berlin.

Stachowicz JJ, Terwin JR, Whitlatch RB, Osman RW (2002) Linking climate change and biological invasions: Ocean warming facilitates nonindigenous species invasions. Proc Natl Acad Sci USA 99:15497–15500. doi:10.1073/pnas.242437499. PubMed: 12422019.

Stachowicz JJ, Byrnes JE (2006) Species diversity, invasion success, and ecosystem functioning: disentangling the influence of resource competition, facilitation, and extrinsic factors. Mar Ecol-Prog Ser 311:251–262.

Taiz L, Zeiger E (1998) Plant defense. In: Plant physiology Sinauer Associates, Sunderland publ 347-376.

Tavares-Colpas F, Orika-Ono E, Domingos-Rodrigues J, de Souza- Passos JR (2003) Effects of some phenolic compounds on soybean seed germination and seed-borne fungi. Braz Arch Biol Techn 46(2):155-161.

Truu M, Juhanson J, Truu J (2009) Microbial biomass, activity and community composition in constructed wetlands. Sci Total Environ 407:3958–3971.

Turner JB, Friedman EI (1974) Fine structure of capitular filaments in the coenocytic green alga *Penicillus*. J Phycol 10:125–134.

Verlaque M, Durand C, Huisman JM, Boudouresque CF, Le Parco Y (2003) On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). Eur J Phycol 38:325–339.

Wallentinus I, Nyberg CD (2007) Introduced marine organisms as habitat modifiers. Mar Pollut Bull 55:323-332. doi:10.1016/j.marpolbul. 2006.11.010. PubMed: 17207503.

Weidner S, Arnold W, Pühler A (1996) Diversity of uncultured micro-organisms associated with the seagrass *Halophila stipulacea* estimated by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA gene. Appl Environ Microbiol 62:766–771.

Windham L (2001) Comparison of biomass production and decomposition between *Phragmites australis* (common reed) and *Spartina patens* (salt hay grass) in brackish tidal marshes of New Jersey, USA. Wetlands 21:179–188.

Žuljević A, Antolić B, Onofri V (2003) First record of *Caulerpa racemosa* (Caulerpales, Chlorophyta) in the Adriatic Sea. J Mar Biol Assoc UK 83:711–712.